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Table of Content

Isolation of ursolic acid from the leaves of <i>Ocimum lamiifolium</i> collected from Addis Ababa Area, Ethiopia Girma Birhanu	65
Morpho-molecular screening of wheat genotypes for heat tolerance Nihar Ranjan Saha, Md.Tariqul Islam, Md. Monirul Islam and Muhammad Shahidul Haque	71
Genetic diversity among Ethiopian sorghum [<i>Sorghum bicolor</i> (L.) Moench] gene bank accessions as revealed by SSR markers Alemu Tirfessa, Tesfaye Tesso, Asfaw Adugna, Hussein Mohammed and Dan Kiambi	84
Introduction of the exotic breeds and cross breeding of local chicken in Ethiopia and solution to genetic erosion: A review Yonas Kejela	92
Effect of methanolic extract of <i>Dennettia tripetala</i> (pepper fruit) on biomarkers of oxidative stress and lipid peroxidation in type 2 diabetic male wistar rats Nwaeme Ogochukwu, Ude Victor Chibueze, Augustine Ndubuisi, Ugwudike Patrick, Agu Francis, Celestine Ani, Emeka Ugwuishi, Sunday Njoba, Lilian Ndubuisi, Hector Obianyido, Joachin Ezeokeke and Nwachukwu Daniel	99
Effectiveness of solid carriers on multiplication of bradyrhizobium mutant strain and nodulation of cowpea (<i>Vigna unguiculata</i> L) Nwokocha, A. G., Fagbola, O. and Daramola, D. S.	106
Chemical and physico-chemical characterization of seeds of melon cultivars: Valenciano and Pele de Sapo, cultivated in the Amazon Flavio Paulino da Silva, Ismael Montero Fernández, Selvin Antonio Saravia Maldonado, Simone Rodrigues da Silva, Ricardo Santos Alemán, Vany Perpetua Ferraz, Jhunion Abrahan Marcia Fuentes and Bernardo de Morais Linhares	114
Analytical quantification of copper in frogs (<i>Rana tigrina</i>) found from various aquatic habitats Muhammad Nasir, Tariq Mahmood Ansari, Hira Javed, Ghulam Yasin, Aleem Ahmad Khan and Muhammad Shoab	121
Immobilization of the Carioca bean (<i>Phaseolus vulgaris</i> L.) extract on SiO₂-TiO₂ aiming the determination of the herbicide Simazine Francisca das Chagas Sobral Silva Mihos, Emerson Schwingel Ribeiro, Maria Helena Wohlers Morelli Cardoso, Lucia Helena Pinto Bastos, Livia Maria da Costa Silva and Andréa Medeiros Salgado	129

Full Length Research Paper

Isolation of ursolic acid from the leaves of *Ocimum lamiifolium* collected from Addis Ababa Area, Ethiopia

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***Ocimum lamiifolium* is a well known medicinal plant in Ethiopia. Fresh leaves of the plant are squeezed and sniffed to treat colds and coughs and as an eye rinse for eye infections, while crushed leaves are used to arrest nose bleeding. In this study phytochemical investigation on the methanol extracts of this plant collected from Addis Ketema subcity, Addis Ababa. A triterpene, namely ursolic acid, was isolated using column chromatography over silica gel. The structure of this compound was identified using one dimensional Nuclear Magnetic Resonance (NMR) spectroscopic techniques such as proton NMR (¹H NMR), carbon-13 NMR (¹³C NMR) and distortionless enhancement by polarization transfer (DEPT), two dimensional NMR spectroscopic method such as HMBC, HMQC and COSY and literature survey.**

Key words: *Ocimum lamiifolium*, Ethiopia, phytochemical investigation, ursolic acid, Nuclear Magnetic Resonance (NMR) spectroscopy.

INTRODUCTION

Natural products are believed to play vital roles in the physiology and ecology of the plants that produce them, particularly as defense elements against pests and pathogens or as attractants for beneficial organisms such as insect pollinators. Because of their biological activities, some plant natural products have been exploited by human beings as pharmaceuticals, stimulants, and poisons (Hadush et al., 2016). Natural products are organic compounds that are formed by living systems. The elucidation of their structures and their chemistry, synthesis and biosynthesis are major areas of organic chemistry. The biologically active constituents of medicinal, commercial and poisonous plants have been studied throughout the development of organic chemistry. Many of these compounds are secondary metabolites. (Hadush et al., 2016).

People living in the villages of Africa, Asia and other parts of the developing world are forced to resort traditional practitioners and to use traditional medicine for the continued maintenance of their health and to alleviate their diverse sufferings. The World Health Organization (WHO) estimated about 80% of the people in the developing countries relies on traditional medicine for primary health care needs, of which a major proportion corresponds to plant extracts (Sarah et al., 2010). Herbal remedies have been used for centuries but more recently, the compounds that are active have been identified, extracted and purified. Synthetic organic chemists have then been able to produce the molecules *in vitro* and so produce them on large scales (Daniel et al., 2012).

Ocimum lamiifolium is a plant which belongs to the

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genus *Ocimum* in the family Lamiaceae and it is an erect, hairy perennial, robust branching shrub or herb growing to 3 m tall. It grows beside roads and streams, in bush land and at forest edges and on grassland between 1200-2900 m (Raimo and Yvonne, 1999). *O. lamiifolium* has been used in folk medicine worldwide since time immemorial. It is used to treat diseases like hepatitis, gonorrhoea, gastric ulcer, asthma and dropsy. It is used as a food additive in Tanzania (Runyoro et al., 2010). In Ethiopia, the plant is known by its vernacular name as 'dama-kassie'. Fresh leaves of *O. lamiifolium* are squeezed and sniffed to treat colds and coughs; and as an eye rinse for eye infections, while crushed leaves are used to arrest nose bleeding. It is also used for the treatment of inflammatory conditions and infections and to treat ailments associated with pyrexia, eye disease, cough, cold, cutaneous leishmaniasis, headache, herpes (kusiil). It is also treated traditionally by squeezing and sniffing fresh leaves of *O. lamiifolium* (Runyoro et al., 2010).

In Ethiopia there are 12 *Ocimum* species namely *Ocimum stirbeyi*, *Ocimum forskolei*, *Ocimum basilicum*, *Ocimum americanum*, *Ocimum ciricinatum*, *Ocimum jameessi*, *Ocimum spicatum*, *Ocimum cufodontii*, *Ocimum gratissimum*, *Ocimum urticifolium*, *Ocimum tricodon* and *Ocimum lamiifolium* (Edwards et al., 2000). Among these species *O. lamiifolium* and *O. basilicum* are widely used in traditional medicine and as a culinary herb, respectively.

Literature survey showed that the isolation of only a flavonoid which is Quercetin-3-O xylosyl (1''→2'') Galactoside from the *O. lamiifolium* plant found in Ethiopia (Grayer, 2002). To the best of my knowledge no other phytochemical investigation was reported. In this thesis research attempt will be made to isolate phytochemicals from the leaves of *O. lamiifolium*. The main objective of this work is to undertake phytochemical study on the methanol extract of the leaves of *O. lamiifolium* and to elucidate structures of the components of the methanol extract of the plant. The plant was selected for this study because of limited study on the phytochemical constituents of the plant and importance of the plant in traditional medicine in Ethiopia.

MATERIALS AND METHODS

Phytochemical investigation

General

¹H, ¹³C, DEPT and 2D NMR experiments were recorded using a Bruker 400 MHz Advance NMR spectrometer in CDCl₃ and deuterated methanol. Thin layer chromatography (TLC) was performed on a 0.25 mm thick silica gel GF₂₅₄ (Merck). Melting points were recorded using Thomas HOOPER capillary melting point apparatus. Components of the *O. lamiifolium* on TLC were detected by their UV fluorescence and by spraying with 1% vanillin in H₂SO₄.

Plant material

Leaves of *O. lamiifolium* were collected from a home grown plant around Addis Ketema subcity, Addis Ababa. A voucher specimen (AAOL001) was deposited at the National Herbarium (ETH), Department of Biology, Addis Ababa University, Addis Ababa.

Isolation and analysis

990 g of powdered leaf of *O. lamiifolium* was first extracted with petroleum ether. The marc from the extract was then soaked with methanol.

Methanol extract

40 g of the crude extract from methanol was applied to a silica gel (251 g) column chromatography and eluted with a solvent system changing continuously from pet. Ether: EtOAc (9:1) to pet. Ether: EtOAc (4:6) and finally with net ethyl acetate and net methanol. 34 fractions were collected and TLC analysis was made. According to their TLC profile, the 34 fractions were reduced to 5 fractions. This is made by comparison of their R_f value, fractions having similar R_f value are mixed.

RESULTS AND DISCUSSION

Phytochemical investigation

Ground aerial parts (990 g) of *O. lamiifolium* were subjected to exhaustive extraction successively with petrol ether, chloroform and methanol. The solvent from each extract was recovered under reduced pressure using a rotavapor to obtain a petrol extract (PE, 54 g), a chloroform extract (CE, 52 g) and a methanol extract (ME, 40 g) respectively. Chromatographic purification of the methanol extract gave a compound coded OLM-1. The structure of this compound has been elucidated on the basis of spectroscopic evidences and in comparison with literature data for similar compounds. Characterizations of compound OLM-1

Compound OLM-1 was a pale yellow crystalline solid with a melting point of 250-251°C obtained from the methanol extract and its characterization was determined using spectroscopic techniques. The COSY spectrum did not give much information as most of the hydrogens are overlapping in the chemical shift region of δ 1.26-1.71 ppm. So it was impossible to unambiguously assign all the signals for each hydrogen by this method. Therefore, most of the NMR assignments were based on HMBC correlations. Only unambiguously assigned signals were taken into consideration when determining this structure. These correlations are shown in Table 1.

The ¹H spectrum (Table 1) indicated the presence of quaternary methyl groups [(δ 0.79, 0.86, 0.99, 1.13, 3H each, s) and (0.87 and 0.95, 3H each, d)]. A downfield doublet integrated for one proton at δ 5.24 coupled with the methylene multiplet at δ 1.93 was assigned to the methine proton on the olefinic carbon. A downfield

Table 1. ^1H , ^{13}C and COSY ($^1\text{H}\leftrightarrow^1\text{H}$) NMR data of compound 1 (in MeOH, δ in ppm).

C no.	δ_{C}	δ_{H}	COSY
C ₁	38.45	1.68	H ¹ ↔H ²
C ₂	26.51	1.64	H ² ↔H ¹ , H ² ↔H ³ , H ² ↔H ²⁰
C ₃	78.31	3.16	H ³ ↔H ²
C ₄	39.17	-	-
C ₅	55.35	0.75	H ⁵ ↔H ⁶
C ₆	18.09	1.37	H ⁶ ↔H ⁵
C ₇	32.95	1.54	-
C ₈	39.39	-	-
C ₉	46.24	1.56	H ⁹ ↔H ¹¹ , H ⁹ ↔H ¹⁵
C ₁₀	36.78	-	-
C ₁₁	22.67	1.93	H ¹¹ ↔H ⁹ , H ¹¹ ↔H ¹² , H ¹¹ ↔H ¹⁵
C ₁₂	125.50	5.24	H ¹² ↔H ¹¹
C ₁₃	138.23	-	-
C ₁₄	41.84	-	-
C ₁₅	27.82	1.09	H ¹⁵ ↔H ⁹ , H ¹⁵ ↔H ¹² , H ¹⁵ ↔H ¹⁶
C ₁₆	23.93	2.03	H ¹⁶ ↔H ¹⁵ , H ¹⁶ ↔H ²²
C ₁₇	46.97	-	-
C ₁₈	52.97	2.20	H ¹⁸ ↔H ¹⁹
C ₁₉	39.03	1.36	H ¹⁹ ↔H ¹⁸
C ₂₀	38.61	1.67	H ²⁰ ↔H ²
C ₂₁	30.38	1.49	H ²¹ ↔H ²²
C ₂₂	36.72	1.00	H ²² ↔H ²¹
C ₂₃	27.36	0.99	H ²³ ↔H ²⁴
C ₂₄	14.94	0.79	H ²⁴ ↔H ²³
C ₂₅	15.002	0.95	-
C ₂₆	16.27	0.86	-
C ₂₇	23.14	1.13	-
C ₂₈	180.21	-	-
C ₂₉	16.33	0.87	-
C ₃₀	20.19	0.95	-

doublet of doublet at δ 3.16 was assigned for the methine proton on the carbon which bears the hydroxyl group. Five other methine protons appeared at δ 0.75 (1H, d, $J_{5,6} = 11.4\text{Hz}$), 1.36 (1H, m), 1.56 (1H, m), 1.67 (1H, m), 2.20 (1H, $J_{18,19} = 11.4\text{ Hz}$). Nine CH₂ methylene protons appeared at δ 1.00 (1H, dd, $J_{21,22} = 3.6\text{ Hz}$, 13.2 Hz), 1.09 (1H, m), 1.37 (1H, m), 1.49 (1H, m), 1.54 (1H, m), 1.57 (1H, m), 1.68 (1H, m), 1.93 (1H, m) and 2.03 (1H, m). The ^{13}C NMR and DEPT-135 (Table 2) indicated that OLM-1 has a total of 30 carbon atoms : seven methyls (δ_{C} 15.00, 16.27, 16.33, 14.97, 20.19, 23.14, 27.36), nine sp³ methines (δ_{C} 38.45, 39.03, 47.61, 52.97, 55.35, 78.31), one sp² methine (125.50), five quaternary sp³ carbons (δ_{C} 39.17, 37.78, 39.39, 41.84, 48.25) and two quaternary sp² carbon (138.23, 180.21). The three sp² carbons (δ_{C} 125.5, 138.23, and 180.21) indicate a double bond functionality and carbonyl. (Figure 1) was proposed for compound OLM-1 from the spectroscopic data

obtained for the compound and in comparison with published data for similar compound (Runyoro et al., 2010). The 2D NMR spectra of OLM-1 further supported the proposed structure (Figure 1).

The positions of hydrogens on carbons were deduced from the HMQC correlation spectrum. The 2D $^1\text{H}\leftrightarrow^1\text{H}$ COSY spectrum showed correlation between the methine proton on C-3 with the methylene proton C-2. In the ^1H NMR spectrum, the hydrogens at δ 3.16 showed multiplicity of doublet of doublet with coupling constant of 4.2 and 11.4 Hz. The methylene hydrogen at δ 1.64 also showed COSY correlation with the methylene hydrogen at δ 1.67(δ 38.45).

In the COSY spectrum, the methylene hydrogens on C-11 showed correlations with the methine hydrogen on C-12. In the ^1H NMR spectrum, the methine hydrogen at δ 5.24(C-12) showed multiplicity of triplet with a coupling constant of δ 3.6 Hz. The chemical shift of the methine

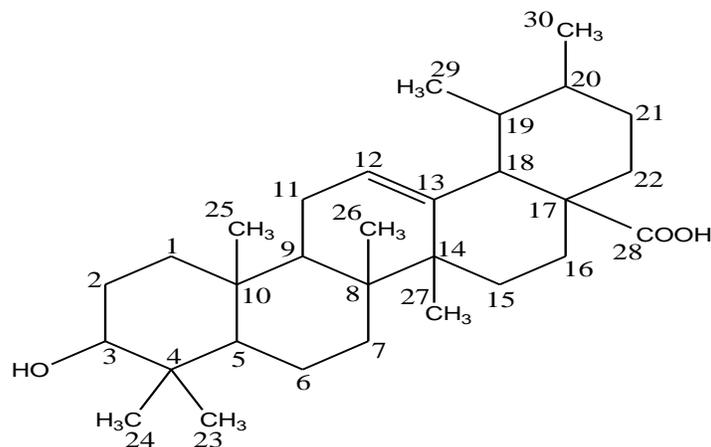


Figure 1. Proposed structure of OLM-1.

Table 2. HMBC ($^1\text{H} \leftrightarrow ^{13}\text{C}$) NMR correlation data of compound 1.

C	δ_c	HMBC
C ₁	38.45	C ¹ ↔ H ² , H ⁴ , H ⁵
C ₂	26.51	H ² ↔ C ¹ , C ⁴ , C ¹⁰
C ₃	78.31	H ³ ↔ C ²³ , C ²⁴
C ₄	39.17	C ⁴ ↔ H ² , H ²³ , H ²⁴
C ₅	55.35	H ⁵ ↔ C ¹ , C ⁶ , C ⁹
C ₆	18.09	C ₆ ↔ H ⁵
C ₇	32.95	C ⁷ ↔ H ²⁶
C ₈	39.39	C ⁸ ↔ H ¹⁵ , H ²⁶ , H ²⁷
C ₉	46.24	H ⁹ ↔ C ¹ , C ⁵ , C ⁸ , C ¹¹ , C ¹⁴ , C ²⁵ , C ²⁶
C ₁₀	36.78	C ¹⁰ ↔ H ²⁵
C ₁₁	22.67	H ¹¹ ↔ C ⁸ , C ⁹ , C ¹² , C ¹³ , C ¹⁴ , C ¹⁸
C ₁₂	125.50	H ¹² ↔ C ⁹ , C ¹¹ , C ¹⁴ , C ¹⁸
C ₁₃	138.23	C ¹³ ↔ H ¹⁵ , H ¹⁸
C ₁₄	41.84	C ¹⁴ ↔ H ¹² , H ¹⁸
C ₁₅	27.82	H ¹⁵ ↔ C ¹⁷
C ₁₆	23.93	H ¹⁶ ↔ C ¹⁸ , C ²⁸
C ₁₇	46.97	C ¹⁷ ↔ H ²¹ , H ²²
C ₁₈	52.97	H ¹⁸ ↔ C ¹⁰ , C ²⁹
C ₁₉	39.03	H ¹⁹ ↔ C ²⁰
C ₂₀	38.61	C ²⁰ ↔ H ¹⁹
C ₂₁	30.38	C ²¹ ↔ H ³⁰
C ₂₂	36.72	H ²² ↔ C ¹
C ₂₃	27.36	H ²³ ↔ C ³ , C ⁴ , C ⁵ , C ²⁴
C ₂₄	14.94	H ²⁴ ↔ C ³ , C ⁴ , C ⁵ , C ²³
C ₂₅	15.002	H ²⁵ ↔ C ⁵ , C ⁹
C ₂₆	16.27	H ²⁶ ↔ C ⁷ , C ⁸ , C ⁹ , C ¹⁰ , C ¹⁴
C ₂₇	23.14	H ²⁷ ↔ C ⁸ , C ¹³ , C ¹⁴ , C ¹⁵
C ₂₈	180.21	C ²⁸ ↔ H ¹⁸ , H ²⁶
C ₂₉	16.33	H ²⁹ ↔ C ¹⁸ , C ¹⁹
C ₃₀	20.19	H ³⁰ ↔ C ¹⁹ , C ²¹

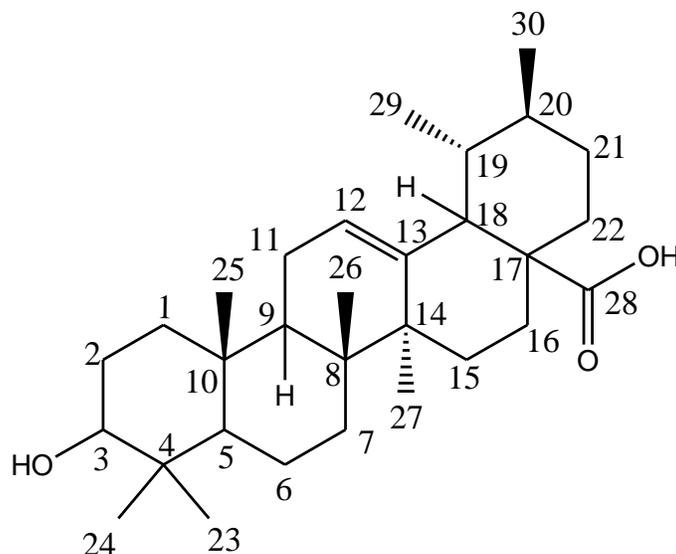


Figure 2. Structure of ursolic acid.

carbon at δ 125.50 indicated that it must be attached to the sp^2 quaternary carbon at δ 138.23. The methine proton on C-19 showed COSY correlation with the methine proton C-18. In the 1H NMR spectrum, the methine hydrogen on C-18 showed multiplicity of doublet with coupling constant 11.4Hz. This indicated that both the methine hydrogens on C-18 and C-19 are in an axial-axial relationship (Table 1).

In the HMBC spectrum (Table 2) the quaternary carbon signal appearing at δ 39.39 showed correlation with the methylene hydrogen on C-11, methyl hydrogens on C-26 and C-27. The quaternary carbon signal appearing at 41.84 had HMBC correlations with the methine hydrogens on C-12 and C-18 indicating the position of C-26 methyl group is likely to be on C-8. The quaternary carbon signal appearing at δ 180.21 showed HMBC correlations with methane hydrogen on C-18 and the methylene hydrogen on C-16 indicating that C-28 is attached to C-17. HMBC correlations were also observed between the methine hydrogen on C-18 with C-10 and C-29. This indicated that C-29 is likely to be attached to C-10. This is supported by the multiplicity of doublet in the 1H NMR spectrum. The methine carbon signal appearing at δ_c 36.78 showed HMBC correlation with the methyl hydrogen on C-30. HMBC correlations were also observed between the methyl hydrogens on C-30 with the methylene carbons C-21 and C-22. In the 1H NMR spectrum, the C-30 methyl hydrogen showed multiplicity of doublet, which indicates that it is attached to C-20. The 1H and ^{13}C NMR data obtained for OLM-1 is comparable with the data for Ursolic acid reported in literature. Based on spectroscopic data and literature survey the compound OLM-1 is characterized to be ursolic acid (Figure 2 and Table 3).

Spectral data

Compound OLM-1: pale yellow crystalline solid, 1H NMR; δ 0.75 (1H, d, H-5), 0.79 (3H, s, Me-24), 0.86 (3H, s, Me-26), 0.87 (3H, d, Me-29), 0.95 (3H, s, Me-27), 0.95 (3H, s, Me-25), 0.99 (3H, s, Me-23), 1.00 (1H, dd, H-22 β), 1.09 (1H, m, H-15 β), 1.13 (3H, s, Me-27), 1.32 (1H, m, H-21 α), 1.34 (1H, m, H-7 β), 1.36 (1H, m, H-19), 1.37 (1H, m, H-6 β), 1.49 (1H, m, H-21 α), 1.54 (1H, m, H-7 α), 1.54 (1H, m, H-6 α), 1.56 (1H, m, H-9), 1.57 (1H, m, H-2 β), 1.64 (1H, m, H-2 α), 1.64 (1H, m, H-1 β), 1.64 (1H, m, H-16 β), 1.67 (1H, m, H-22 α), 1.67 (1H, m, H-20), 1.68 (1H, m, H-1 α), 1.93 (1H, m, H-11 α), 1.93 (1H, m, H-11 β), 1.93 (1H, m, H-15 α), 2.03 (1H, td, H-16 α), 2.20 (1H, d, H-18), 3.16 (1H, dd, H-3), 5.24 (1H, t, H-12); ^{13}C NMR, 14.94 (C-24), 15.002 (C-25), 16.27 (C-26), 16.33 (C-29), 18.09 (C-6), 20.19 (C-30), 22.67 (C-11), 23.14 (C-27), 23.93 (C-16), 26.51 (C-2), 27.36 (C-23), 27.82 (C-15), 30.38 (C-21), 32.95 (C-7), 36.72 (C-22), 36.78 (C-10), 38.45 (C-1), 38.61 (C-20), 39.03 (C-19), 39.17 (C-4), 39.39 (C-8), 41.84 (C-14), 46.24 (C-9), 46.97 (C-17), 52.97 (C-18), 55.35 (C-5), 78.31 (C-3), 125.50 (C-12), 138.23 (C-13), 180.21 (C-28).

Conclusions

In this thesis research the methanol extract results one triterpen; ursolic acid (OLM-1). This compound was identified by comparing thesis 1H and ^{13}C NMR spectroscopic data for similar compounds from literature.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

Table 3. Comparison of ^{13}C NMR of compound OLM-1 with that of ursolic acid.

Carbon	^{13}C NMR compound OLM-1	^{13}C NMR Ursolic acid [80]
1	38.45	39.8
2	26.51	27.8
3	78.31	79.6
4	39.17	39.9
5	55.35	56.7
6	18.09	19.4
7	32.95	34.3
8	39.39	40.7
9	46.24	47.6
10	36.78	38.1
11	22.67	24.3
12	125.50	126.8
13	138.23	139.6
14	41.84	42.8
15	27.82	27.36
16	23.93	25.3
17	46.97	47.6
18	52.97	54.3
19	39.03	40.4
20	38.61	40.4
21	30.38	31.7
22	36.72	38.1
23	27.36	28.7
24	14.94	16.0
25	15.002	16.3
26	16.27	17.6
27	23.14	24.0
28	180.21	181.6
29	16.33	17.8
30	20.19	21.5

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Full Length Research Paper

Morpho-molecular screening of wheat genotypes for heat tolerance

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Wheat (*Triticum aestivum* L.) production in Bangladesh is often impaired by heat stress. Therefore, it has been a priority to develop heat tolerant wheat variety for Bangladesh. An investigation was carried out to evaluate locally cultivated wheat genotypes for heat tolerance based on morpho-physiological and molecular markers. A pot experiment was carried out with ten locally cultivated wheat genotypes. Heat treatment was imposed, 5 days after anthesis, in a plant growth chamber at 35°C and 70% RH for 3 days. The heat stress affected all the yield contributing characters and ultimately led to a reduction in grain yield. BARI gom-29, BARI gom-30 and BARI gom-28 emerged as heat tolerant variety on the basis of susceptibility index and tolerance efficiency. While, Shatabdi, BARI gom-23, BARI gom-26 and BARI gom-24 were heat susceptible. Twenty six wheat genotypes were screened for heat tolerance through seven linked SSR markers that generated 44 alleles among the 26 wheat genotypes with an average of 6.28 alleles per locus. Overall polymorphism information content (PIC) and Nei's gene diversity were 0.68 and 0.72, respectively. Similarity indices based unweighted pair group method with arithmetic mean (UPGMA) analysis separated 26 genotypes into five different clusters. Two morphologically identified tolerant genotypes namely BARI gom-29, BARI gom-30 and one moderate genotype BARI gom-22 were grouped in cluster 2. Therefore, these three varieties can be suitable for cultivation in the north-western part of Bangladesh as heat tolerant cultivars.

Key words: Diversity, heat tolerance efficiency, heat susceptibility index, SSR marker.

INTRODUCTION

Wheat (*Triticum aestivum* L.) has a prominent position among the cereals and supplements nearly one-third of the total world population's diet by providing half of the dietary protein and more than half of the calories (Kasana et al., 2016). During the last four decades of the 20th century, the global wheat production is doubled from 3 to

6 billion and by the year 2020 demand for wheat imposed by growing population is forecasted around 950 million tonnes (Kailash et al., 2017). This target will be achieved only if global wheat production is increased by 2.5% per annum (Singh et al., 2011). This increase in wheat production is much more challenging due to a shortage of

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water and changing climate.

Seasonal fluctuations have a potential impact on the crop development and grain yield. The variation in temperature requirements and temperature extremes varies widely for different cultivars of the same species, among species and it varies widely for most crops. Kalra et al., (2008) emphasized the need of studying the response of crops to weather variations for evaluating the impact of seasonal temperature change and estimating yield dependence of temperature rise of crops. Too early sowing of crop produced weak plants with poor root system as the temperature is above optimum whereas delay in sowing leads to irregular germination which results in poor tillering and finally reduction in yield (Yajam and Madani, 2013). Many authors have reported a reduced crop stand, shorter life cycle, reduced tillering, less biomass production, reduced fertilization and grain development, reduced head size, reduction in number of spikes per plant, number of grains per spike and grain weight as the consequences of heat stress, and all these changes are translated in reduction of grain yield/m² under heat stress conditions (Moshatati et al., 2012).

Wheat is very sensitive to high temperature (Slafer and Satorre, 1999) and trends in increasing growing season temperatures have already been reported for the major wheat-producing regions (Alexander et al., 2006; Gaffen and Ross 1998; Hennessy et al., 2008). Wheat experiences heat stress to varying degrees at different phenological stages, but heat stress during the reproductive phase is more pronounced than during the vegetative phase due to the direct effect on grain number and dry weight (Wollenweber et al., 2013).

Yield and yield components in stress condition, are still the most effective tools for stress evaluation (Ozkan et al., 1998). For exploitation of genetic variations to improve stress tolerance and development of stress tolerant cultivars, plant breeders mainly relies on selection of different genotypes under environmental stress conditions (Khan et al., 2014). In spite of several screening methods in many crops and development of selection criteria by different researchers, very few were reported for screening heat tolerant genotypes in wheat. Stress indices based on loss of yield under stress conditions in comparison to normal conditions have been used for screening stress tolerant genotypes. Stress susceptibility index (SSI) was proposed as a ratio of genotypic performance under stress and non-stress conditions and was suggested for measurement of yield stability that apprehended the changes in both potential and actual yields in variable environments (Fischer and Maurer, 1978). Bansal and Sinha (1991) proposed to use SSI and grain yield/m² as stability parameters to identify drought resistant genotypes of wheat. Sood et al., (2017) used SSI to distinguish between wheat. With this in mind, it was felt imperative to evaluate some improved wheat genotypes facing high temperatures during and after anthesis under field conditions to identify genotypes that have high yield potential in both relatively favourable and

high-temperature environments for using in a breeding program.

Marker-assisted selection (MAS) approaches have contributed greatly to a better understanding of the genetic bases of plant stress-tolerance in some crops (Liu et al., 2006; Momcilovic and Ristic, 2007) that led to the enhanced tolerance to abiotic stresses. Synthesis of low molecular weight HSP's (heat shock proteins) synthesis in *T. durum* and the response of different heat tolerant *T. aestivum* genotypes to the enzymes like NRA and Peroxidase can reliably indicate thermo-tolerance. Because of the general complexity of abiotic stress tolerance and the difficulty in phenotypic selection, MAS is considered as an effective approach to improve this kind of tolerance. Sadat et al., (2013) revealed the utility of SSR marker linked with various heat tolerant traits like grain filling duration, Heat Susceptibility Index (HSI)/single kernel weight of main spike, HIS/grain filling duration and HSI/kernel weight under heat stress in MAS for screening wheat genotypes to heat stress. However, limited research has been done to identify genetic markers associated with heat tolerance in different plants and no such efforts have been made in Bangladesh. Thus, there is an urgent need to understand genetic factors affecting heat tolerance as well as to identify new diagnostic markers to be deployed in MAS, which will ensure faster yield gains under heat stress environments. In the present investigation, several locally cultivated wheat genotypes were evaluated with the aim to find heat tolerance based on morph-physiological traits and molecular markers.

MATERIALS AND METHODS

Plant materials collection for morphological and molecular screening

Twenty five germplasm were originally collected from Regional Wheat Research Centre (RWRC), Rajshahi and one germplasm (BINA gom-1) was collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, (Table 1). Ten genotypes for morphological screening were selected on the basis of their performance in the experiments of a preliminary screening in the previous year (Billah, 2017). The morphological screening experiment was carried out at the net house, Crop Physiology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh during the period from November 2017 to March 2018. The molecular experiment was carried out at the Molecular Biology Laboratory; Department of Biotechnology, Bangladesh Agricultural University (BAU), Mymensingh.

Morphological screening of wheat for heat tolerance

Pot preparation

A bulk volume of soil was collected, sun dried, ground and sieved. All kinds of weeds, stubbles and residues of crop and weeds were removed. Each of the pots was filled with 10 kg homogeneous soil. Urea, Muriate of Potash (MP), Triple Super Phosphate (TSP) and Bio-fertilizer were applied according to Fertilizer Recommendation

Table 1. List of wheat genotypes for morphological and molecular screening.

S/N	Germplasm name	Year of release	Pedigree
1	BARI gom- 25*	2010	ZSH 12/HLB 19//2*NL297
2	BINA gom- 1	2016	-
3	Aghrani	1987	INIA/3/SN64/P416OE//SN64
4	Akbar	1983	RON/TOB or ROBIN-M/(SIB)TOBARI-66
5	Sourav*	1998	NAC/VEE
6	BARI gom- 20*	1998	TURACO/CHIL
7	Shatabdi	2000	MRNG/BUC//BLO/PVN/3/PJB-81
8	BARI gom- 22*	2005	KAN/6/COQ/F61.70//CNDR/3/OLN/4/PHO/5/MRNG/ALDAN//CNO
9	BARI gom- 23*	2005	NL297*2/LR25
10	BARI gom- 24*	2005	G-162/BL-1316//NL-297
11	BARI gom- 26*	2010	ICTAL123/3/RAWAL87//VEE/HD2285
12	BARI gom- 27	2012	Waxwing*2/Vivitsi
13	BARI gom- 28*	2012	CHIL/2*STAR/4/BOW/CROW//BUC/PVN/3/2*VEE#10
14	BARI gom- 29*	2014	-
15	BARI gom- 30*	2014	-
16	BARI gom- 33	2017	-
17	Barkat	1983	BB/GLL//CARP/3/PVN or BLUEBIRD/GALLO//CARPINTERO/3/(SIB)PAVON-76
18	Durum	-	<i>Triticumturgidum</i> L.
19	KalayanSona	1968	PJ/GB55 or PENJAMO-62(SIB)/GABO-55
20	Kanchan	1983	UP301/C306
21	Kheri	-	-
22	Pavon- 76	1979	VCM//CNO/7C/3/KAL/BB [VICAM-71//CIANO-67/SIETE-CERROS-66/3/KALYANSONA/BLUEBIRD]
23	Protiva	1993	KU SELECTION 12
24	Sonalika	1973	II53.388/AN//YT54/N10B/3/LR/4/B494.A.4.18.2.IY/Y53//3*Y50 or II53-388/ANDES//SIB/PITIC-62/3/LERMA-ROJO-64
25	Sonora- 64	1968	YAKTANA-54//NORIN-10/BREVOR/3/2*YAQUI-54
26	Triticale	2009	-

Here, the names with asterisks (*) were used in morphological study.

Guide (FRG, 2017).

Experimental design

The experiment was laid out in a completely randomized design (CRD) with three replications. Thus the total number of pots were 60 (10×3×2) for this experiment. Seven seeds were sown in each pot at a depth of one inch. After successful germination, only three plants were left in each pot as the extra plants were removed.

Heat stress treatment

After 5 days of anthesis, a set of pots were subjected to heat treatment in plant growth chamber (VS-91G09M-1300C). All of 10 varieties were kept in growth chamber for 3 days at 35°C with 70% RH. After the heat stress, pots were returned to the experimental field where the non-treated plants were kept.

Morphological characters

The plant height, length of flag leaf, width of flag leaf and flag leaf

area were measured and number of leaves per plant was recorded from three plants of each pot before harvesting and mean value was calculated. Leaf chlorophyll content was recorded by using a portable chlorophyll meter (SPAD-502, Minolta, Japan) and photosynthetic rate was measured from the flag leaf of the plant by portable photosynthesis system (Li-6400XT, LI-COR, USA).

The number of effective tillers was recorded at physiological maturity, the spike length, number of spikelet per spike, numbers of filled and unfilled grains per spike, numbers of filled and unfilled grain per spikelet, number of grains per plant, grain weight per plant, 1000-grain weight, shoot weight, total dry matter and days to harvest were recorded after harvesting from three plants of each pot and mean value of three plants was calculated and used to analyse.

Harvest index (HI %)

The harvest index was calculated from three days oven dried plant sample according to the following rules:

$$HI (\%) = \frac{\text{Grain yield/Plant}}{\text{Biological yield/Plant}} \times 100$$

Heat tolerance efficiency

Heat tolerance efficiency (HTE) for total grain yield per plant was calculated by the following formula:

$$\text{HTE (\%)} = \frac{\text{Yield under stress condition/Plant}}{\text{Yield under control condition/Plant}} \times 100$$

Heat susceptibility index

Heat susceptibility Index (HSI) based total grain yield per plant was calculated by the following formula as suggested by Fischer and Maurer (1978).

$$\text{HSI} = \left(1 - \frac{\text{YS}}{\text{YC}}\right) / \left(1 - \frac{\text{XS}}{\text{XC}}\right)$$

Here, YS=Yield under stress condition (g), YC=Yield under control condition (g), XS=Mean yield of all genotypes under stress condition and XC=Mean yield of all genotypes under control condition.

Molecular screening of wheat for heat tolerance

DNA extraction

Genomic DNA was isolated from 21-day old green leaves using CTAB method with minor modifications (IRRI). Purified DNA was checked for quality and quantity using agarose gel electrophoresis as well as Nano Drop spectrophotometer (Thermo Scientific, www.nanodrop.com). Finally, diluted DNA (50 ng/μl) was used to amplify DNA by SSR markers using eppendorf thermo-cycler. The SSR profiles of the amplified products of five representative primers are shown in Figure 2(A-E).

SSR marker genotyping

Thirteen SSR markers linked to heat tolerance were used in screening for heat tolerance wheat variety. Primer name, sequences and corresponding annealing temperatures are listed (Table 2). The polymerase chain reaction (PCR) cocktail including DNA had total volume of 10 μl/reaction (IRRI standard protocol) for SSR analysis, composed of 1.0 μl genomic DNA, 5 μl PCR master mix (Go-taq green master mix, Promega corporation, U.S.A), 0.5 μl forward primer, 0.5 μl reverse primer, and 3 μl nuclease free water. Samples were subjected to the following thermal profile for amplification in a thermo cycler: The reaction mix was preheated at 94°C for 3 min followed by 35 cycles of 30 s. denaturation at 94°C, 45 sec annealing at 55-65°C (based on the annealing temperature of the individual primer) and elongation at 72°C for 2 min. After the last cycle, a final step was maintained at 72°C for 7 min to allow complete extension of all amplified fragments followed by holding at 4°C until electrophoresis.

Visualization of amplification products was accomplished on 8% Polyacrylamide gel in 1 X TAE buffer. The Polyacrylamide gel was stained with ethidium bromide solution for 20-25 min. The stained Polyacrylamide gel was illuminated by UV-trans-illuminator and photographed for assessing the DNA profiles. Only five representative gel pictures have been given in this paper to represent allelic variation at DNA level.

Data analysis

The data obtained in the morphological study were statistically analyzed using analysis of variance (ANOVA) and least significance difference (LSD). The mean was separated by Duncan's Multiple Range Test (Gomez and Gomez, 1984) using MSTAT-C software. Molecular weights of PCR products were estimated using AlphaEaseFC 4 software and the number of alleles per locus, major allele frequency, genetic diversity and polymorphism information content (PIC) values were determined with the help of a genetic analysis software, POWER MARKER version 3.23 (Liu and Muse, 2005). The allele frequency data from POWER MARKER was used to export the data in binary format (allele presence = "1" and allele absence = "0") for analysis with NTSYS-PC version 2.1 (Rolf 1997). The genetic similarity was calculated using 0/1 matrix and SIMQUAL subprogram (Nei and Li, 1979). The resultant similarity matrix helped to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based unweighted pair group method with arithmetic mean (UPGMA) as implemented in NTSYS-PC (version 2.1) (Rolf, 1997) to infer genetic relationships and phylogeny. For estimating the similarity matrix, null alleles were treated as missing data to reduce the biased genetic or similarity measures (Warburton and Crossa, 2002).

RESULTS AND DISCUSSION

Morphological screening of wheat for heat tolerance

An artificial temperature controlled facility (Plant growth chamber; VS-91G09M-1300C) was used to simulate the thermal environment in the present study. Similar treatment methods have been reported in other studies because of its better environmental control (Rehman et al., 2009; Feng et al., 2014). It was observed in this study that heat shock resulted in negative impact on all the morphological and physiological characters, except for the number of unfilled grain spike⁻¹ and spikelet⁻¹ which increased due to heat stress (Tables 3 to 5). Mohammadi et al., (2004) reported the effects of post anthesis heat stress on head traits of wheat.

The combined analysis of variance showed significant effect for the source of variation for all traits, indicating that the heat stress influenced the expression of the traits. Grain yield decreased from 10.14 g in favourable conditions, to 7.003 g in the stress condition; hence, an average reduction of 30.99% was estimated (Table 5). In the present study, a grain yield reduction from 17.79 to 57.43% was found (Table 6). However, grain yield reduction ranged from 60 to 95% is reported (Albrecht et al., 2007; Yildirim and Bahar, 2010).

The overall mean of yield components also decreased as a function of the heat stress. The number of grains spike⁻¹ and spikelet⁻¹, number of spikelet spike⁻¹ and 1000-grain weight was highly affected by the heat stress. All genotypes had the largest decrease for the component number of grains spike⁻¹. Number of grains spike⁻¹ seemed to be the most affected trait by the heat stress. The reduction in the number of grains spike⁻¹ can be attributed to the heat effect on the differentiation of floral organs, male and female sporogenesis, pollination

Table 2. List of the selected primers used for heat tolerance screening in wheat genotypes.

Primer name		Sequence	A _T (°C)	Amplified band (bp)	References
Xbarc84-3B	F	CGCATAACCGTTGGGAAGACATCTG	64	123	Billah (2017)
	R	GGTGCAACTAGAACGTACTTCCAGTC			
gwm132-6B	F	TACCAAATCGAAACACATCAG G	60	116-118	Najeb et al. (2011)
	R	CATATCAAGGTCTCCTTCCCC			
Xgwm285-3B	F	ATGACCCTTCTGCCAAACAC	60	223	Billah (2017)
	R	ATCGACCGGGATCTAGCC			
Xgwm428-3A	F	AGCGTTCTTGGGAATTAG AGA	60	133-137	Najeb et al. (2011)
	R	CCAATCAGCCTGCAACAA C			
Xgwm577-7B	F	ATGGCATAATTTGGTGAATT G	55	136-222	Najeb et al. (2011)
	R	TGTTTCAAGCCCAACTTCTATT			
Xgwm617-6A	F	GATCTT GCGCTGAGAGAGA	60	133	Najeb et al. (2011)
	R	CTCCGATGGATTACTCGCAC			
Xbarc121-7A	F	ACTGATCAGCAATGTCAACTGAA	55	68-221	Najeb et al. (2011)
	R	CCGGTGTCTTTCTAACGCTATG			

and fertilization (Farooq et al., 2011). High temperatures affect pollen viability, reducing the number of fertilized flowers (Rahman et al., 2009). Similar results were observed by Yildirim and Bahar (2010), the number of grains spike⁻¹ decreased from 33 in the ideal condition of cultivation to 13 in heat stress condition. At the same heat stress condition, the grain weight reduced from 43 to 14 g. In our study the number of grains spike⁻¹ decreased from 48.61 in the ideal condition of cultivation to 37.32 in heat stress condition. Under the same conditions, the grain mass reduced from 10.14 to 7.003 g (Table 5).

Reduction in grain weight between 21 and 35% due to heat was reported by Assad and Paulsen (2002). Later, Shah and Paulsen (2003) found that the reduction under stress results from the decrease in the photosynthetic rate of the flag leaf and early leaf senescence. In addition to the damage caused to photosynthesis, starch deposition in grain reduced because the enzymes involved in the biosynthesis of starch are sensitive to high temperatures (Denyer et al., 1994). The yield decrease (19.89 to 57.43%) encountered under heat stress in the present study might be due to the reduction of photosynthetic rate (Tables 3 and 6). One of the main reasons for the deleterious effect of high temperatures is the photosynthesis inhibition (Taiz and Zeiger, 2004). Consequently, carbohydrate reserves dropped and organs lost sugars, causing decrease in production.

Effect of heat treatment on heat tolerance parameter of wheat

There was significant interaction between genotypes and

environments for the grain weight. This indicates that the genotypes have different performance when subjected to different environments. For instance, different genotypes express different degrees of heat tolerance. A practical approach to identifying heat tolerant genotypes is to use tolerance indices, which measure the ability of genotypes to maintain their productive potential in stress conditions.

The heat susceptibility index is used in wheat breeding programs for heat tolerance (Khanna-Chopra and Viswanathan, 1999; Rahman et al., 2009; de Oliveira et al., 2011). The reduction in performance when sown under heat-stress conditions from that of the optimum environment was calculated. HSI<1 indicates the tolerance of genotype to heat stress, whereas HSI>1 indicates susceptibility of the genotypes under stress (Fischer and Maurer, 1978). The comparison of these values was used to identify genotypes with least susceptibility to thermal stress. The heat tolerance as measured by heat susceptibility index reflects the stability of performance of genotypes under control and heat stress environments and does not take into account the actual yield obtained under heat stress (Simarjit et al., 2009).

Heat susceptibility index values for the grain weight per plant ranged from 0.57 to 1.86 in the present study. The cultivars BARI gom-28, BARI gom-29, BARI gom-30, BARI gom-25, BARI gom-20 and BARI gom-22 were relatively heat resistant (HSI values <1) and they exhibited smaller yield reductions under heat stress compared with optimum conditions than the mean of all genotypes. On the contrary, the varieties Shatabdi, BARI gom-23, BARI gom-26 and BARI gom-24 were relatively heat susceptible (HSI >1) with concomitant higher yield

Table 3. Effect of interaction of heat treatment (35 °C) and genotype on morpho-physiological characteristics.

Genotype	Treatment	PH	NTP	NLP	LFL	WFL	FLA	CC	PR
Shatabdi	Control	79.71 ^{fg}	15.89 ^a	36.78 ^a	21.61 ^g	1.911 ^{bc}	39.19 ^{e-g}	45.48 ^{bc}	58.47 ^{cd}
	35°C	72.12 ^h	10.89 ^{d-f}	21.89 ^{e-h}	16.70 ^h	1.711 ^{e-g}	27.17 ^m	32.90 ⁱ	53.22 ^e
BARI gom-23	Control	79.78 ^{fg}	13.22 ^b	21.22 ^{f-h}	18.57 ^h	1.911 ^{bc}	33.67 ^k	40.13 ^{f-h}	61.94 ^{ab}
	35°C	79.56 ^{fg}	8.223 ^k	15.33 ^{ij}	17.89 ^h	1.800 ^{c-f}	30.61 ^l	39.83 ^{gh}	53.92 ^e
BARI gom-26	Control	86.02 ^{b-d}	12.45 ^{bc}	26.00 ^{de}	29.52 ^a	1.900 ^{b-d}	52.83 ^a	42.18 ^{d-f}	63.30 ^a
	35°C	83.00 ^e	8.000 ^k	14.22 ^{i-k}	26.20 ^{b-d}	1.934 ^{bc}	47.67 ^b	39.60 ^{gh}	53.73 ^e
BARI gom-28	Control	87.61 ^{ab}	12.11 ^{bc}	34.56 ^{ab}	26.70 ^{bc}	1.722 ^{e-g}	42.99 ^{cd}	46.40 ^b	64.67 ^a
	35°C	81.84 ^{ef}	9.557 ^{g-j}	17.78 ^{hi}	23.78 ^{d-g}	1.655 ^{f-h}	37.33 ^{g-j}	39.23 ^h	54.50 ^e
BARI gom-29	Control	86.56 ^{a-c}	11.78 ^{cd}	31.00 ^{bc}	27.49 ^{ab}	1.856 ^{b-e}	48.00 ^b	50.99 ^a	63.22 ^a
	35°C	78.34 ^g	9.667 ^{g-i}	14.89 ^{ij}	22.57 ^{fg}	1.700 ^{e-g}	36.57 ^{h-j}	41.39 ^{e-h}	55.55 ^{de}
BARI gom-30	Control	88.64 ^a	11.67 ^{c-e}	25.89 ^{de}	25.82 ^{b-e}	1.667 ^{f-h}	40.72 ^{d-f}	44.17 ^{cd}	64.53 ^a
	35°C	81.90 ^{ef}	10.00 ^{f-h}	14.78 ^{ij}	23.83 ^{d-g}	1.522 ^h	34.67 ^{jk}	42.80 ^{de}	54.33 ^e
BARI gom-25	Control	88.11 ^{ab}	9.890 ^{f-h}	29.89 ^{cd}	24.34 ^{c-f}	1.978 ^b	46.73 ^b	43.10 ^{de}	54.30 ^e
	35°C	86.00 ^{b-d}	8.113 ^k	14.00 ^{i-k}	21.43 ^g	1.967 ^b	40.00 ^{ef}	41.48 ^{e-g}	41.51 ^g
BARI gom-24	Control	84.33 ^{c-e}	8.890 ^{h-k}	25.11 ^{ef}	26.22 ^{b-d}	2.222 ^a	55.00 ^a	45.79 ^{bc}	52.87 ^e
	35°C	78.71 ^g	8.777 ^{h-k}	15.67 ^{ij}	23.33 ^{e-g}	1.989 ^b	44.33 ^c	42.87 ^{de}	45.89 ^f
BARI gom-20	Control	86.79 ^{a-c}	10.55 ^{e-g}	22.89 ^{e-g}	23.30 ^{e-g}	1.745 ^{d-g}	38.87 ^{f-h}	44.37 ^{b-d}	59.29 ^{bc}
	35°C	83.77 ^{de}	9.000 ^{h-k}	12.44 ^{jk}	23.36 ^{e-g}	1.600 ^{gh}	35.09 ^{i-k}	43.07 ^{de}	55.06 ^e
BARI gom-22	Control	89.00 ^a	8.557 ^{i-k}	18.67 ^{g-i}	24.83 ^{c-f}	1.778 ^{c-f}	41.62 ^{de}	41.56 ^{e-g}	55.95 ^{de}
	35°C	88.16 ^{ab}	8.333 ^{jk}	10.11 ^k	23.13 ^{fg}	1.800 ^{c-f}	39.72 ^{e-g}	33.80 ⁱ	55.11 ^e
	P-value	**	**	**	*	**	**	**	**
	LSD(0.05)	2.279	1.095	4.186	3.207	0.138	2.387	1.969	3.082
	LSD(0.01)	3.049	1.465	5.602	3.087	0.185	3.195	2.634	4.124
	CV (%)	1.65	6.46	11.99	5.94	4.64	3.56	2.84	3.33

In a column, figures with same letter (s) or without letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT). **, * = Significant at 1% and 5%, respectively level of probability, PH= Plant height (cm), NTP= No. of tiller plant⁻¹, NLP= No. of leaf plant⁻¹, LFL= Length of flag leaf (cm), WFL= Width of flag leaf (cm), FLA= flag leaf area (cm²), DB= Days to booting, CC= Chlorophyll content (SPAD reading) and PR= Photosynthetic rate ($\mu\text{CO}_2\text{cm}^{-2}\text{s}^{-1}$).

reduction. Significant differences were observed in HTE and HSI in all the genotypes under stress condition. The HTE in susceptible genotypes ranged from 42.57 to 64.28% while in tolerant genotypes; it ranged from 71.71 to 82.21%. HSI ranged from 1.16 to 1.86 in the susceptible genotypes and 0.57 to 0.91 in tolerant genotypes. Among the susceptible genotypes BARI gom-26 (1.86) had a higher HSI whereas lower HIS was found in BARI gom-24 (1.16). Among the tolerant genotypes, BARI gom-25 had the highest HSI (0.915) and BARI gom-29 had the lowest (0.575).

On the basis of above discussion, under heat stress, the variety BARI gom-28, BARI gom-29, BARI gom-30, BARI gom-25, BARI gom-20 and BARI gom-22 emerged as tolerant to heat based on HSI and HTE. Therefore, these genotypes had low heat susceptibility indicating their specific suitability under late sowing condition. These results are in conformity with those of Khan et al. (2014) concurred that some genotypes have potential to produce high yield even under high temperature. Among these cultivars, BARI gom-29 showed the highest grain yield followed by the cultivars BARI gom-30 and BARI gom-28

Table 4. Effect of interaction of heat treatment (35 °C) and genotype on yield contributing characters.

Genotype	Treatment	TDM	NSP	NSLS	LS	NGS	NUS
Shatabdi	Control	39.37 ^a	5.557 ^c	21.44 ^{bc}	11.72 ^b	48.89 ^{a-c}	11.56 ^{c-e}
	35°C	32.24 ^{cd}	3.667 ^{f-i}	21.33 ^{bc}	11.50 ^{bc}	33.78 ^{gh}	12.34 ^{cd}
BARI gom-23	Control	39.34 ^a	4.890 ^{cd}	20.00 ^{b-e}	11.14 ^{b-e}	40.33 ^{d-f}	12.55 ^{cd}
	35°C	28.48 ^e	3.890 ^{e-i}	19.11 ^{c-g}	11.04 ^{b-f}	28.33 ^h	13.00 ^{bc}
BARI gom-26	Control	33.44 ^c	4.667 ^{c-e}	18.78 ^{d-g}	10.38 ^{c-g}	52.33 ^a	8.443 ^{fg}
	35°C	27.80 ^e	3.443 ^{hi}	16.67 ^g	9.713 ^{gh}	32.56 ^{gh}	10.78 ^{de}
BARI gom-28	Control	33.66 ^c	6.443 ^b	18.67 ^{d-g}	9.733 ^{f-h}	48.44 ^{a-c}	9.887 ^{e-g}
	35°C	27.38 ^e	3.000 ^{ij}	18.00 ^{e-g}	8.887 ^h	32.45 ^{gh}	11.11 ^{c-e}
BARI gom-29	Control	34.81 ^{bc}	7.333 ^a	19.44 ^{b-f}	11.40 ^{b-d}	47.33 ^{a-c}	11.11 ^{c-e}
	35°C	27.43 ^e	4.337 ^{d-h}	18.78 ^{d-g}	10.20 ^{c-g}	34.67 ^{fg}	12.67 ^{cd}
BARI gom-30	Control	38.93 ^a	7.000 ^{ab}	18.44 ^{d-g}	10.69 ^{b-g}	50.11 ^{ab}	8.333 ^{fg}
	35°C	29.06 ^e	4.447 ^{d-g}	18.11 ^{e-g}	10.54 ^{b-g}	37.55 ^{e-g}	11.67 ^{c-e}
BARI gom-25	Control	38.17 ^a	4.667 ^{c-e}	20.89 ^{b-d}	10.96 ^{b-g}	42.78 ^{c-e}	12.56 ^{cd}
	35°C	27.36 ^e	4.443 ^{d-g}	19.89 ^{b-f}	10.83 ^{b-g}	37.78 ^{e-g}	15.00 ^a
BARI gom-24	Control	39.96 ^a	4.553 ^{d-f}	23.67 ^a	13.69 ^a	53.78 ^a	11.78 ^{c-e}
	35°C	28.94 ^e	2.190 ^j	20.11 ^{b-e}	11.08 ^{b-e}	44.33 ^{b-d}	14.55 ^{ab}
BARI gom-20	Control	34.36 ^c	4.780 ^{c-e}	18.11 ^{e-g}	10.07 ^{e-h}	48.56 ^{a-c}	8.113 ^g
	35°C	30.22 ^{de}	3.557 ^{g-i}	17.44 ^{fg}	10.14 ^{d-g}	44.44 ^{b-d}	10.11 ^{ef}
BARI gom-22	Control	37.36 ^{ab}	4.443 ^{d-g}	21.45 ^{bc}	10.46 ^{b-g}	53.55 ^a	10.22 ^{ef}
	35°C	30.12 ^{de}	3.223 ⁱ	21.67 ^{ab}	10.08 ^{d-h}	47.33 ^{a-c}	12.33 ^{cd}
	P-value	**	**	*	*	**	*
	LSD(0.05)	2.771	0.837	2.085	1.111	5.679	1.709
	LSD(0.01)	3.708	1.119	2.791	1.486	7.599	2.286
	CV (%)	5.1	11.2	6.45	6.28	8.01	9.08

In a column, figures with same letter (s) or without letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT). **, * = Significant at 1% and 5%, respectively level of probability, TDM= Total dry matter (g), NSP= No. of spike plant⁻¹, NSLS= No. of spikelet plant⁻¹, LS= Length of spike (cm), NGS= No. of grain spike⁻¹ and NUS= No. of unfilled grain spike⁻¹.

with better adaptation to heat. In addition to be more productive, these varieties showed the higher number of tillers per plant, photosynthetic rate, harvest index and low mass reduction for grain yield under heat condition. Billah (2017) found that BARI gom-29 had superior performance under adverse conditions, recommending its cultivation in unfavourable environments. In contrast, Shatabdi, BARI gom-23, BARI gom-26 and BARI gom-24 preformed as susceptible varieties under heat stress condition. Because of these varieties had showed lower yield due to higher yield reduction under stress condition. BARI gom-25, BARI gom-20 and BARI gom-22 varieties

were intermediate in their performance under heat stressed condition. The study revealed that there are significant differences in performance among genotypes in regard to each trait.

Molecular screening of wheat for heat tolerance

Overall SSR diversity

Data derived from these experiments were analyzed to evaluate the usefulness of the microsatellites for genetic

Table 5. Effect of interaction of heat treatment (35 °C) and genotype on yield contributing characters.

Genotype	Treatment	NGSL	NUSL	NGP	GW	TGW	HI
Shatabdi	Control	2.957 ^{b-e}	0.680 ^f	263.3 ^{a-c}	11.26 ^b	42.90 ^{b-d}	28.60 ^d
	35°C	2.303 ^g	0.691 ^f	160.8 ^{hi}	6.843 ^{gh}	38.74 ^e	21.26 ^{ef}
BARI gom-23	Control	2.527 ^{fg}	0.692 ^f	176.6 ^{gh}	8.293 ^{ef}	44.10 ^{a-c}	20.83 ^{e-g}
	35°C	1.893 ^{hi}	0.801 ^{c-e}	127.2 ^j	4.507 ^{jk}	26.30 ^h	15.74 ^h
BARI gom-26	Control	3.357 ^a	0.534 ^{hi}	233.9 ^{c-e}	9.700 ^c	41.55 ^{b-e}	28.33 ^d
	35°C	1.923 ^{hi}	0.822 ^{a-c}	175.2 ^{gh}	4.130 ^k	25.26 ^h	14.87 ^h
BARI gom-28	Control	3.187 ^{ab}	0.639 ^{fg}	274.3 ^{ab}	11.96 ^{ab}	45.33 ^{ab}	35.04 ^a
	35°C	1.687 ⁱ	0.912 ^a	188.4 ^{f,h}	9.163 ^{c-e}	28.92 ^{gh}	33.42 ^{ab}
BARI gom-29	Control	3.100 ^{a-c}	0.712 ^{ef}	264.0 ^{a-c}	11.82 ^{ab}	47.51 ^a	33.33 ^{ab}
	35°C	1.987 ^h	0.916 ^a	167.6 ^h	9.717 ^c	26.94 ^{gh}	35.00 ^a
BARI gom-30	Control	3.197 ^{ab}	0.575 ^{g-i}	287.6 ^a	12.56 ^a	40.59 ^{c-e}	32.03 ^{bc}
	35°C	2.550 ^{fg}	0.908 ^{ab}	178.6 ^{gh}	9.457 ^{cd}	30.84 ^{fg}	32.33 ^{bc}
BARI gom-25	Control	2.560 ^{fg}	0.721 ^{d-f}	154.1 ^{h,j}	7.527 ^{fg}	43.20 ^{bc}	19.33 ^g
	35°C	1.850 ^{hi}	0.873 ^{a-c}	131.0 ^{ij}	5.397 ^{ij}	29.31 ^{gh}	19.54 ^{fg}
BARI gom-24	Control	3.057 ^{bc}	0.621 ^{f-h}	253.4 ^{bc}	12.48 ^a	48.14 ^a	30.67 ^c
	35°C	2.723 ^{ef}	0.810 ^{b-d}	239.2 ^{cd}	8.023 ^{ef}	34.36 ^f	27.73 ^d
BARI gom-20	Control	3.027 ^{bc}	0.518 ⁱ	202.9 ^{e-g}	7.357 ^{fg}	38.86 ^{de}	21.38 ^{ef}
	35°C	2.750 ^{d-f}	0.571 ^{g-i}	181.2 ^{gh}	5.983 ^{hi}	28.66 ^{gh}	19.83 ^{fg}
BARI gom-22	Control	2.997 ^{b-d}	0.566 ^{g-i}	219.4 ^{d-f}	8.417 ^{d-f}	38.66 ^e	22.51 ^e
	35°C	2.860 ^{c-e}	0.705 ^{ef}	177.2 ^{gh}	6.810 ^{gh}	28.96 ^{gh}	22.59 ^e
	P-value	**	**	**	**	**	**
	LSD(0.05)	0.245	0.09	30.38	1.055	3.729	1.714
	LSD(0.01)	0.328	0.121	40.65	1.412	4.99	2.294
	CV (%)	5.68	8.01	9.08	7.46	6.2	4.04

In a column, figures with same letter (s) or without letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT). **, * = Significant at 1% and 5%, respectively level of probability, NGSL= No. of unfilled grain spikelet⁻¹, NUSL= No. of unfilled grain spikelet⁻¹, NGP= No. of grain plant⁻¹, GW= Grain weight plant⁻¹(g), TGW= 1000-Grain weight (g) and HI= Harvest index.

diversity and screening of heat tolerance of the 26 wheat varieties. The 7 SSRs produced a total of 44 alleles ranging from 2 to 10 with an average of 6.28 alleles per marker. Markers Xgwm577 produced the highest number of alleles (10), whereas the Xgwm428 produced the lowest number of alleles (2) (Table 7). This finding agrees with earlier results of Prasad et al., (2000) and Amer et al., (2001). Such variation in the number of allele amplified by different primer sets is attributable to several factors including primer structure and number of annealing sites in the genome (Kernodle et al., 1993). Obviously, polymorphic bands revealing differences

among genotypes would be used to examine and establish systematic relationships among genotypes as reported by Hadrys et al. (1992).

Polymorphic information content (PIC) values were estimated as a measure of genetic diversity among the genotypes. A PIC higher than 0.5, between 0.5 and 0.25 and less than 0.25, has been used as scale for loci polymorphism to be considered high, medium or low, respectively (Vaiman et al., 1994). In the current study, PIC values ranged from 0.33 for Xgwm428 to 0.87 for Xgwm577, with an average of 0.68 per marker (Table 7). Hence, the PIC values recorded in this study are high.,

Table 6. Effect of heat treatment on heat tolerance parameter of wheat.

Genotype	Decrease (%) (NGS)	Decrease (%) (TDM)	Decrease (%) (GW)	HTE	HSI
Shatabdi	30.91	18.394	39.225	60.775	1.269
BARI gom-23	29.75	39.744	45.640	54.360	1.476
BARI gom-26	37.79	26.619	57.429	42.571	1.858
BARI gom-28	33.03	29.634	23.393	76.607	0.757
BARI gom-29	26.76	45.205	17.790	82.210	0.575
BARI gom-30	25.06	38.276	24.715	75.285	0.799
BARI gom-25	11.69	32.744	28.292	71.708	0.915
BARI gom-24	17.56	32.363	35.724	64.276	1.156
BARI gom-20	8.47	37.028	18.640	81.360	0.603
BARI gom-22	11.62	43.710	19.049	80.951	0.616

Here, NGS= No. of grain spike⁻¹; TDM= Total dry matter; GW= Grain weight plant⁻¹; HTE= Heat tolerance efficiency and HSI = Heat susceptibility index.

Table 7. Summary statistics of 7 SSR markers found among 26 wheat genotype.

Marker name	Allele no.	Rare allele	Null allele	Major allele		Gene diversity	PIC
				Frequency	Size (bp)		
Xbarc84	7	1	0	0.2692	125	0.8107	0.7845
Xgwm132	5	1	0	0.3846	119	0.7278	0.6824
Xgwm285	8	-	0	0.1923	213	0.8609	0.8449
Xgwm428	2	-	0	0.6923	191	0.4260	0.3353
Xgwm577	10	1	0	0.1923	136	0.8817	0.8701
Xgwm617	8	1	0	0.2692	116	0.8284	0.8075
Xbarc121	4	2	2	0.5769	160	0.5444	0.4619
Mean	6.285	0.857	0.285	0.3681	151	0.7257	0.6838

and significantly higher than the PIC values reported from other studies Roder et al. (1995) and Plaschke et al (1995) but Uddin and Boerner (2008) found similar observations. The markers showed an average PIC values of 0.68 which confirm that SSR markers used in this study were highly informative because PIC values higher than 0.50 indicate high polymorphism. According to Saghai-Marouf et al., (1984), markers with PIC values of 0.5 or higher are highly informative for genetic studies. The PIC can be looked as the measurement of usefulness of each marker in distinguishing one individual from another. The PIC values and rare alleles are proved to be useful information in genetic diversity analysis of genotypes. The simple sequence repeats (SSRs) represent the most suitable marker system in wheat (Hammer et al., 2000) and have been successfully used to characterize genetic diversity in advanced wheat breeding materials Dreisigacker (2004).

Genetic similarity analysis using weighted pair group method of arithmetic mean (UPGMA)

A dendrogram was constructed based on the Nei's

(1973) genetic distance calculated from the 44 SSR alleles (by 7 SSR Primer) generated from 26 wheat genotypes. All 26 wheat genotypes could be easily distinguished. The UPGMA cluster analysis showed significant genetic variation among the wheat genotype studied, with a similarity coefficient varying between 0.13 and 0.86. The UPGMA cluster analysis led to the grouping of the 26 germplasm into five major clusters formed at 0.33 cut off similarity coefficient below which the similarity values narrowed conspicuously. All the clusters were subdivided into two sub clusters (Figure 1). The cluster-1 consisted with ten genotypes, of which one tolerant (BARI gom-28), two moderately tolerant (BARI gom-20 and BARI gom-25) and two susceptible genotypes (BARI gom-23 and Shatabdi). Similarly the cluster-2 grouped with five genotypes, of which two tolerant (BARI gom-29 and BARI gom-30), one moderately tolerant (BARI gom-22) and one susceptible (BARI gom-26) genotype as well as another cluster-4 was contained a susceptible genotype (BARI gom-24). These clusters were contained also some genotypes which was not included in phenotypic study in our experiment. The phenotypically studied tolerant, moderately tolerant and susceptible genotypes were

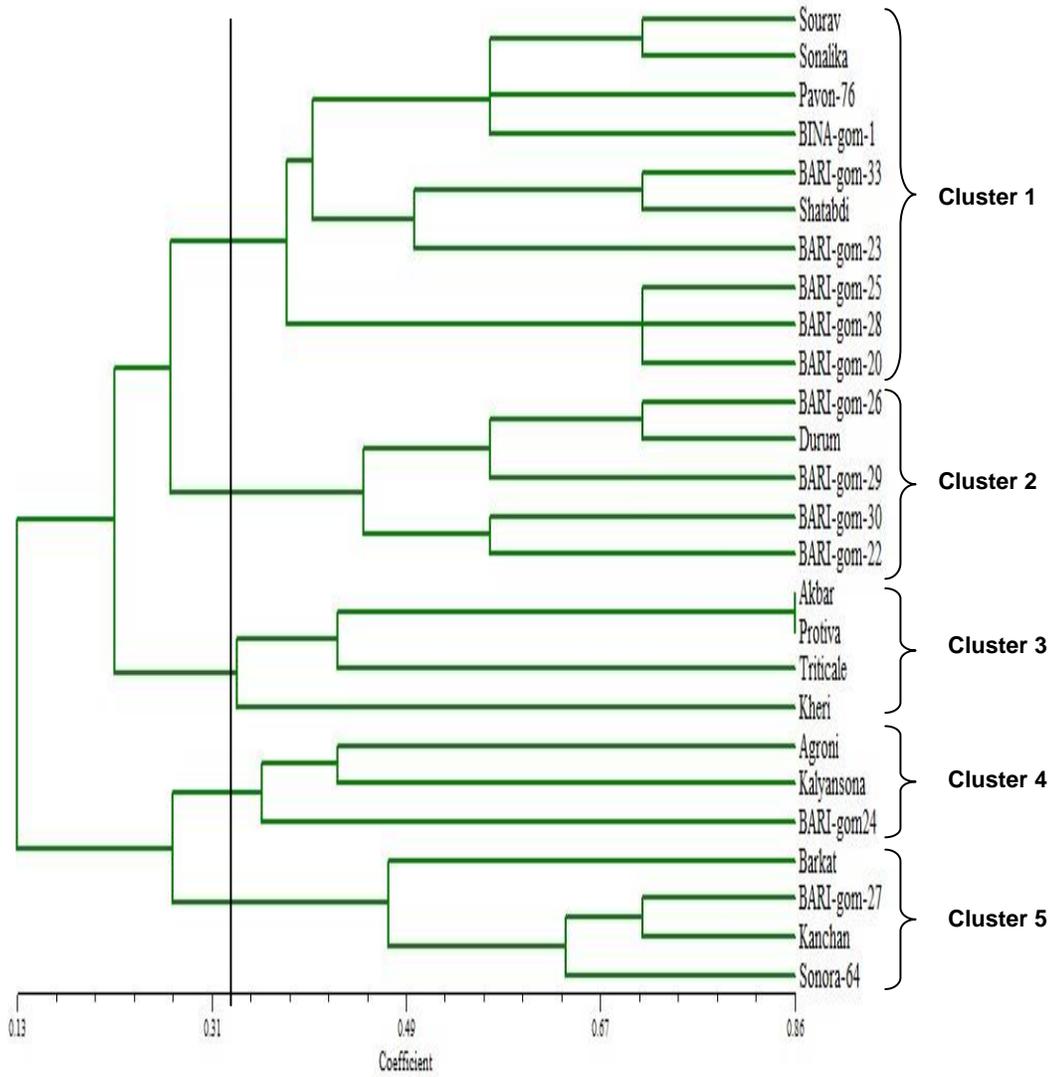


Figure 1. UPGMA cluster for 26 Wheat genotypes showing the genetic diversity and relatedness among them.

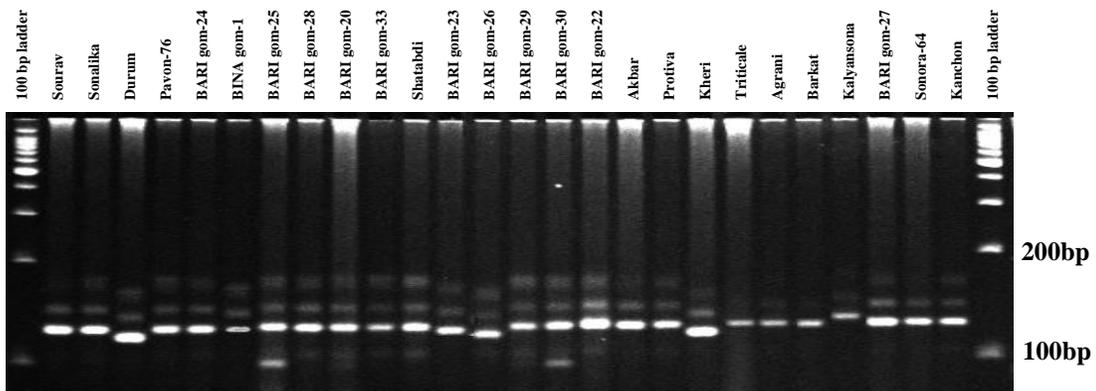


Figure 2a. Banding pattern of allele at locus Xbarc84 in 26 wheat genotypes (wells 2-27). Wells 1 and 28 are 100 bp ladders.

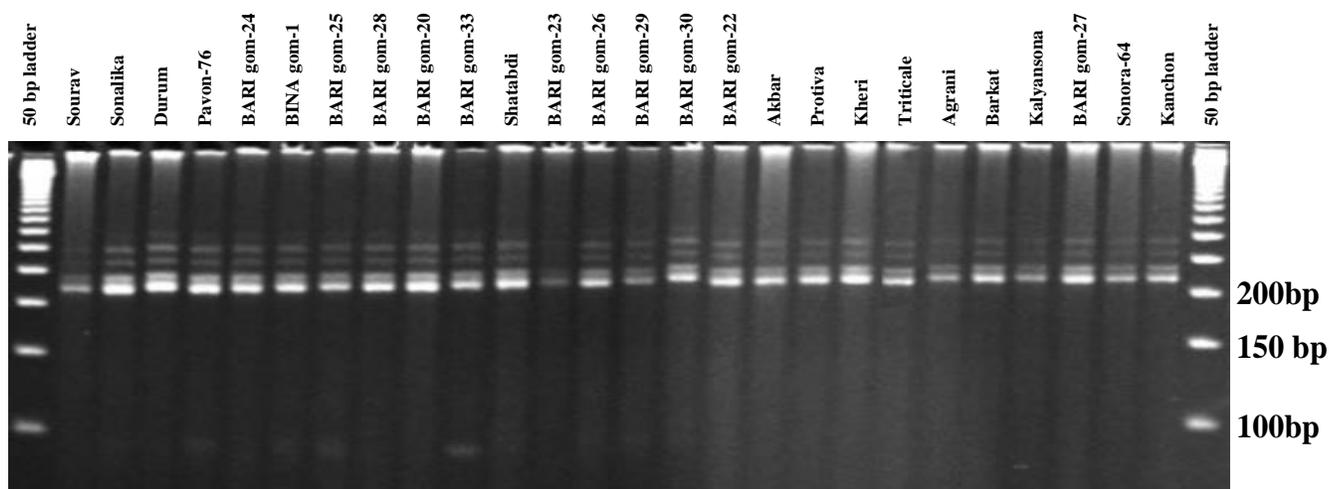


Figure 2b. Banding pattern of allele at locus Xgwm285 in 26 wheat genotypes (wells 2-27). Wells 1 and 28 represent 50 bp ladders.

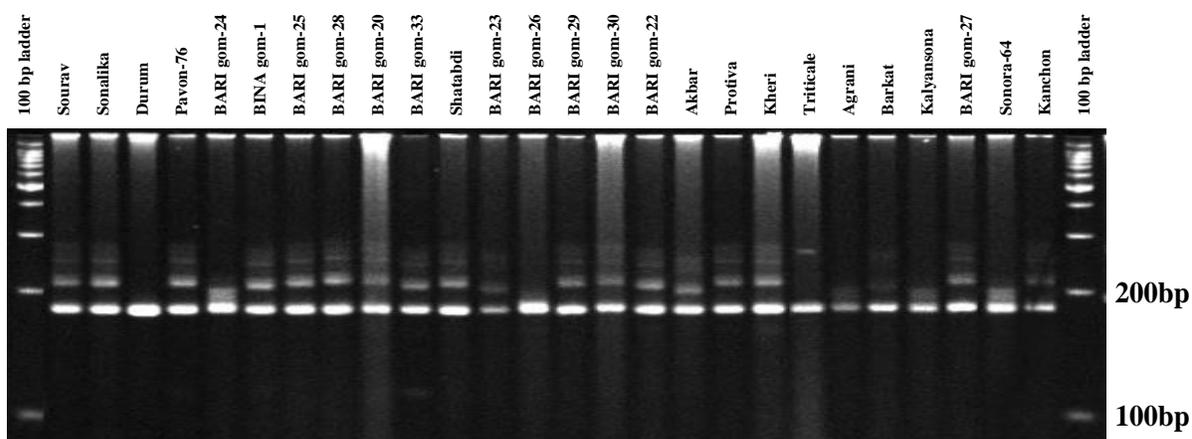


Figure 2c. Banding pattern of allele at locus Xgwm428 in 26 wheat genotypes (wells 2-27). Wells 1 and 28 are 100 bp ladders.

randomly present in the three clusters (1, 2 and 4). The reason for their inclusion in this same cluster is obscure. The potential of these genotypes to be tolerant to heat needs to be reevaluated in future study. Although the genotypes included in the phenotypic study have been known to be heat tolerant (DHCROP, 2018), our experiment revealed that three of them were tolerant, three were moderately tolerant and rest was susceptible. The marker assisted study revealed a discrimination of the 26 genotypes into 5 clusters. Two tolerant genotypes namely BARI gom-29 and BARI gom-30 and one moderately tolerant genotype BARI gom-22 were in cluster-2. As these three genotypes were found to be tolerant both in morphological and molecular studies of successive two years they can be recommended for cultivation in the north-western part of Bangladesh as

heat tolerant variety.

Conclusion

The results of molecular and physiological characterization were taken under consideration simultaneously. It was observed that the genotypes showed nearly distinct arrangement according to their performance in physiological characterization. Although the genotypes included in the phenotypic study have been known to be heat tolerant, our experiment revealed that three of them were tolerant, three were moderately tolerant and rest were susceptible. The marker assisted study revealed a discrimination of the 26 genotypes into 5 clusters. Two tolerant genotypes namely BARI gom-29

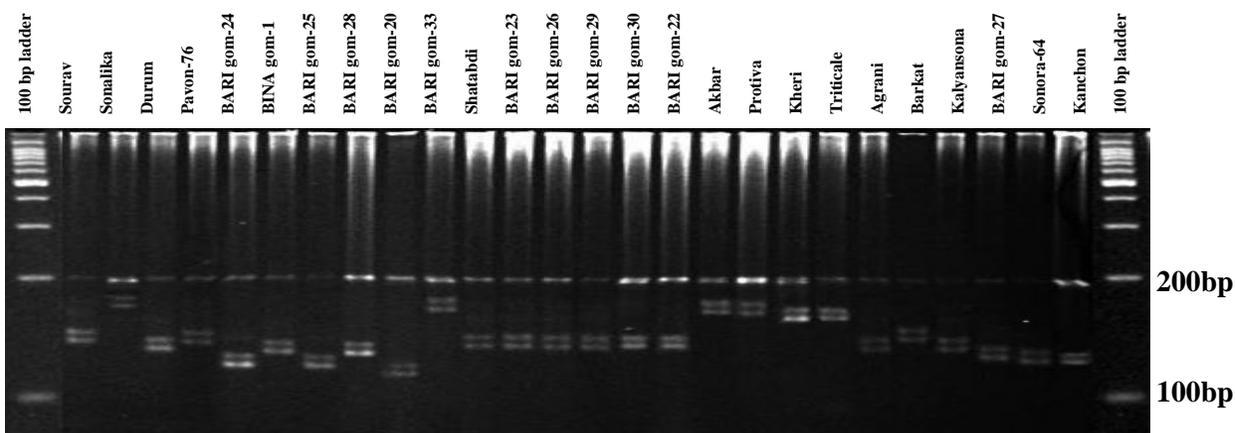


Figure 2d. Banding pattern of allele at locus Xgwm577 in 26 wheat genotypes (wells 2-27). Wells 1 and 28 represent 100 bp ladders.

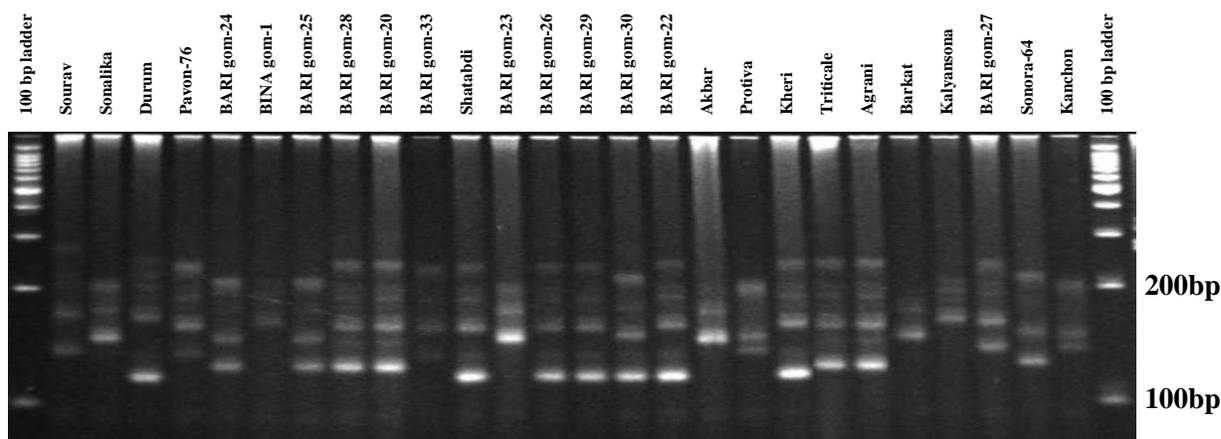


Figure 2e. Banding pattern of allele at locus Xgwm617 in 26 wheat genotypes (wells 2-27). Well 1 and 28 are 100 bp ladders.

and BARI gom-30 and one moderate genotype BARI gom-22 were in cluster-2. As these three genotypes were found to be tolerant both in morphological and molecular studies of successive two years they can be recommended for cultivation in the north-western part of Bangladesh as heat tolerant variety.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity among Ethiopian sorghum [*Sorghum bicolor* (L.) Moench] gene bank accessions as revealed by SSR markers

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The presence of genetic variation in plant populations is useful for conservation and use in breeding programs. This study was conducted to estimate the extent and patterns of genetic diversity among 200 sorghum accessions collected from different parts of Ethiopia and preserved in a gene bank. Using 39 polymorphic simple sequence repeat (SSR) markers, which were previously mapped, 261 alleles were produced with mean 6.7 alleles per SSR. Polymorphism Information Content (PIC) and Dice's similarity coefficient values ranged from 0.06 to 0.81 and from 0.062 to 0.96, respectively. Hierarchical clustering using UPGMA analysis revealed three major clusters with no clear distinction among geographical origins. Moreover, analysis of molecular variance (AMOVA) indicated that the majority of the variation (99.62%) observed was attributed to differences among accessions and only a small fraction of the total variation (0.38%) was related to regions of original collection, which may indicate that geographical origin is not a useful guide to follow for germplasm collection. Rather, agro-ecological classifications may be better for collection mission. Furthermore, crossing of accessions from the three distant clusters could result in promising genotypes for use as varieties or parents for the future breeding programs.

Key words: Allele, cluster, genetic variability, polymorphism information content, sorghum, SSR.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] belongs to the Family Poaceae (the grass family) and the tribe

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Andropogoneae (de Wet, 1978). The species is believed to have originated in northeastern quadrant of Africa, an area currently occupied by Ethiopia, Eritrea and Sudan (FAO, 1995). Evolutionary evidence exists to support this claim such as the wide range of sorghum types cultivated in the region and the diverse form of wild and weedy sorghum species still prevalent in the area (Ejeta and Grenier, 2005; Tesso et al., 2011). All the basic races of sorghum, except kaffir, are believed to be found in Ethiopia though the *durras* make the largest proportion of the race composition (Stemler et al., 1975).

Sorghum is the fifth most important cereal globally and is a staple for millions of people in Africa and South East Asia (Ejeta and Grenier, 2005). It is used for food, feed, construction and bioenergy. In Ethiopia, the grain is used for various purposes including the preparation of traditional foods in the form of different recipes, for the preparation of local beverages, and the straw is used as animal feed, for fuel and for construction. It is the second most important cereal in the country next to tef and maize (CSA, 2012).

Sorghum, in general, has tremendous genetic variability which is particularly evident in areas of early domestication or early introduction of the crop as are the situations of Ethiopia and Asia, respectively. This diversity in sorghum is readily apparent from the diverse morpho types grown in the country and the wide agro-ecological zones coverage of the crop (Doggett, 1988). Thousands of accessions of this species have been collected in the country and deposited in the international gene banks at ICRISAT in India (Reddy et al., 2006) and at USDA-ARS National Plant Germplasm System (NPGS) (Cuevas and Prom, 2013). Few of these have been sources of genes that contributed to some of the major breakthroughs in sorghum improvement programs, including high lysine (*hl*), high protein digestibility (*hpd*), and the stay green genes (Adugna, 2014).

Attempts have been made to estimate genetic diversity among Ethiopian sorghum gene bank accessions (Ayana et al., 2000), *in situ* landrace populations (Adugna, 2014) as well as sorghum germplasm pools from the African region (Agrama and Tuinstra, 2003). Although these studies have generated useful information and contributed to knowledge of sorghum germplasm in the region, further investigation to unravel the extent of genetic variability and establish the pattern of genetic relationship among the accessions is essential. The power of molecular marker technology and the steady improvement of its application will enhance further understanding of the pattern of genetic relationship in sorghum and facilitate its utilization in breeding programs. Therefore, this study was conducted to determine the extent and pattern of genetic variability among the Ethiopian sorghum germplasm accessions using simple sequence repeat markers (SSRs).

MATERIALS AND METHODS

Description of the study areas and plant materials

The sorghum growing environments in Ethiopia are traditionally classified into four broad categories representing the major agro-ecologies. These are the dry and wet lowlands with altitudes of less than 1600 m, the mid-altitude areas with elevation of between 1601 m and 1900 m and the highlands with altitudes of over 1900 m (Gebrekidan, 1981). For the sake of simplicity, the dry and the wet lowland agro-ecologies in this study were merged and represented as "lowland". Figure 1 represents the map of Ethiopia showing the seven sites where the germplasm set was originally collected. The geographical characteristics including altitude, geographical coordinates (latitude and longitude) and agro-ecological classifications are presented in Table 1. A total of 200 sorghum accessions representing seven geographical regions within the above agro-ecological categories were received from the Ethiopian Institute of Biodiversity (EIB) for this study (Table 1, and Supplementary Table S1). The accessions were randomly drawn from over 9000 accessions maintained at EIB during 2008, but the sampling was set to represent the three major agro-ecologies.

DNA extraction, PCR amplification and genotyping

Fifty seeds from each of the 200 sorghum accessions were planted in a pot in the green house at Melkassa Agricultural Research Center, Ethiopia in May 2008. Leaf tissue samples of 15 individual four-week-old seedlings from every accession were collected, placed in medicinal bags containing silica gel, sealed and shipped to the Biosciences for East and Central Africa (BECA) laboratory in Nairobi, Kenya for DNA analysis. Total DNA was extracted from bulk sample of 15 individuals per accession using a modified CTAB protocol.

A total of 39 polymorphic sorghum SSR primer pairs, which were previously mapped, were chosen for genotyping the accessions (Table 2) based on their level of polymorphism and consistent amplification across accessions and genome coverage observed previously. PCR was run either in 96-well or 384-well plates with a total reaction volume of 5 μ L that consisted of 5 ng genomic DNA, 1X magnesium free PCR buffer, and 2.0 mM $MgCl_2$, 2 pmoles of fluorescent dye-labelled forward primer labelled with FAM, VIC, NED and PET fluorescent dyes and 2 pmoles of un-labelled reverse primer, 2 mM of dNTPs, 5 Unit AmpliTaq Gold DNA polymerase (Applied Biosystems).

Touch-down PCR amplifications were performed for each primer pair separately using GeneAmp PCR System 9600 (Applied Biosystems). This consisted of 15 min at 94°C for the initial denaturation followed by ten cycles of 94°C for 10 s, 61°C for 20 s (ramp of 1°C per cycle) and 72°C for 30 s, then by 31 cycles of 94°C for 10 s, 54°C for 20 s and 72°C for 30 s, and a final extension of 20 min at 72°C. After the PCR amplification few samples from each primer pair were randomly selected and checked for proper amplification and product intensity in 2% agarose gels after electrophoresis. PCR products were separated by co-loading post-PCR products based on fluorescent dye and/or fragment size. Depending on band intensity on agarose gel, 0.5 to 1.0 μ L of PCR products from each of the 6-FAM, VIC, NED and PET-labelled PCR products were pooled together and the final volume adjusted to 10 μ L by adding the required volume from a mix of an injection solution (HiDi) and size standard (GS500LIZ) (1 mL HiDi and 12 μ L GS500 LIZ for 384-well plates). DNA fragments were denatured and size-fractionated using ABI 3730 Capillary DNA sequencer (PE-Applied

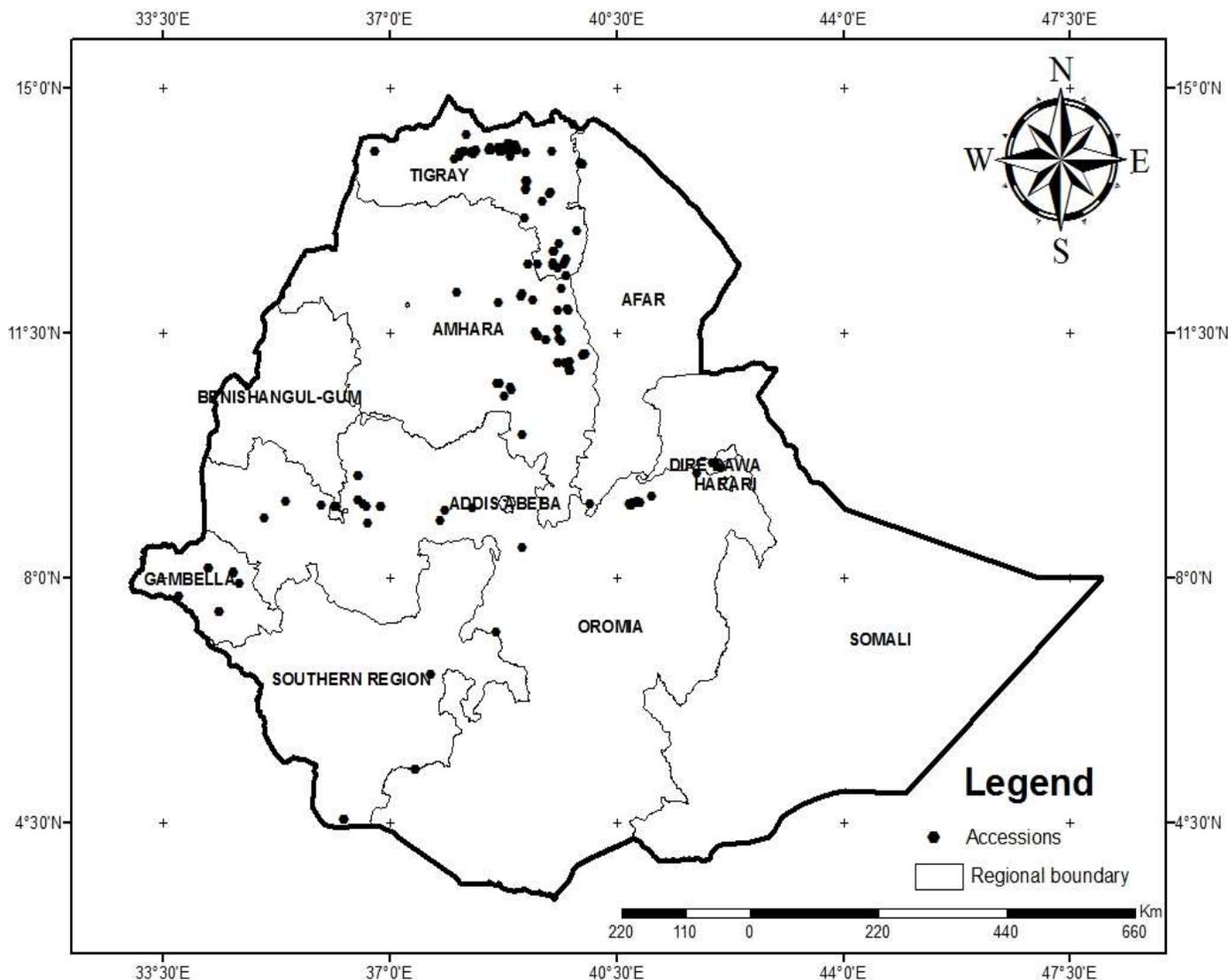


Figure 1. Map of Ethiopia showing the origin of accessions

Biosystems) as described in the user's manual. The peaks were sized and the alleles called using GeneMapper software version 3.7 and the internal size standard GS500LIZ-3730. A positive control sample (genotype BTx623) was included in all PCR for verifying the repeatability of each PCR and genotyping data (allele calls).

Data scoring and statistical analysis

Quality index, total number of alleles, maximum, minimum and abundant alleles (s) for 39 SSR markers were calculated from AlleloBin program. Those markers which showed unacceptable quality index were excluded from final analysis. Since the maximum number of alleles in a given bulked sample was higher than the two alleles expected for a diploid individual, the allele sizes data were converted into "1" and "0" where a "1" indicates the presence of a

specific allele and "0" indicates its absence. Thus, all statistical analyses were performed after converting the adjusted allele calls into binary format.

Similarity coefficient was calculated as a measure of genetic similarity among accessions and used to generate a dendrogram using the hierarchical clustering and Unweighted Pair Group Method using Arithmetic Averages (UPGMA) algorithm of DARwin version 5.0 (Perrier and Jacquemoud-Collet, 2006). Principal coordinate analysis (PCoA) was used to investigate the overall variation and patterns of relationship among the accessions based on the broad agro-ecologies using DARwin. Analysis of molecular variance (AMOVA) was used to partition SSR variation among-groups and among accessions within-groups components. Significance levels for variance component estimates were computed by a non-parametric permutation procedure, using 1000 permutations. AMOVA and F_{ST} indices were calculated using the

Table 1. Geographical characteristics of the regions of collection of the sorghum germplasm accessions used in the study

Region of collection	Altitudinal class	Number of accessions	Altitude range m	Latitude	Longitude
Gamo Gofa	Mid-altitude	3	1600	05°16'	37°23'
	Lowland	7	500-1270	04°11'-06°38'	36°16'-37°38'
Hararghe	Highland	42	2090	09°05'	40°48'
	Mid-altitude	11	1640-1850	09°03'-09°30'	40°42'-41°44'
	Lowland	63	1250-1430	09°35'-09°39'	41°59'-42°06'
Illubabor	Lowland	12	400-600	07°31'-08°09'	33°45'-34°41'
Shewa	Highland	4	1980-2370	08°49'-10°03'	37°47'-39°02'
	Mid-altitude	2	1600-1900	07°14'-08°26'	38°38'-39°02'
Tigray	Highland	37	1920-2485	12°31'-14°50'	38°16'-39°60'
	Mid-altitude	2322	1600-1900	12°20'-14°21'	38°00'-39°59'
	Lowland	16	700-1570	11°57'-14°60'	36°05'-39°50'
Wollega	Highland	1	2260	08°47'	36°39'
	Mid-altitude	78	1600-1880	09°02'-09°40'	35°24'-36°51'
	Lowland	7	1340-1480	08°47'-09°40'	35°04'-36°51'
Wollo	Highland	2421	1930-2540	10°36'-12°30'	34°01'-39°45'
	Mid-altitude	14 17	1600-1880	11°02'-12°09'	39°15'-40°01'
	Lowland	23	1470-1580	10°58'-12°26'	39°25'-39°46'
Total		200			

Table 2. Summary of the 39 SSR markers used to characterize the 200 sorghum accessions in 2008.

Locus	Repeat motif	SBI chromosome	Allele size range bp		PIC	No. of alleles in this study
			Previous studies	This study		
gpsb067	GT10	SBI-08	160-190	168 - 184	0.57	6
gpsb123	CA7+GA5	SBI-08	284-304	282 -294	0.40	5
mSbCIR223	AC6	SBI-02	101-124	103 -115	0.44	4
mSbCIR238	AC26	SBI-02	69-129	73 -111	0.50	10
mSbCIR240	TG9	SBI-08	102-180	105 -201	0.29	4
mSbCIR246	CA7.5	SBI-07	86-114	93 -101	0.52	2
mSbCIR248	GT7.5	SBI-05	79-111	89 -101	0.28	6
mSbCIR262	CATG3.25	SBI-10	208-446	212 -221	0.42	3
mSbCIR276	AC9	SBI-03	221-252	227 -231	0.19	3
mSbCIR283	CT8 GT8.5	SBI-10	111-157	113 -155	0.75	12
mSbCIR286	AC9	SBI-01	104-150	104 -128	0.28	7
mSbCIR300	GT9	SBI-07	74-118	100 -112	0.18	4
mSbCIR306	GT7	SBI-01	118-126	119 -123	0.37	3
mSbCIR329	AC8.5	SBI-05	73-121	105 -115	0.35	5
SbAGB02	AG ₃₅	SBI-07	92-176	92 -126	0.65	9
Xcup02	GCA6	SBI-09	186-216	194 -203	0.16	3
Xcup14	AG ₁₀	SBI-03	209-251	204 - 246	0.34	3
Xcup53	TTTA ₅	SBI-01	182-202	178 -194	0.51	3

Table 2. Contd.

Xcup61	CAG ₇	SBI-03	189-204	195 -198	0.20	2
Xcup63	GGATGC ₄	SBI-02	127-163	141 -147	0.14	2
Xgap072	AG16	SBI-06	168-229	172 -208	0.55	8
Xgap084	AG14	SBI-02	171-235	175 -211	0.73	13
Xgap206	AC13/AG20	SBI-09	86-164	98 -146	0.79	12
XISEP0310	CCAAT4	SBI-02	158-219	179 -204	0.36	2
Xtxp010	CT ₁₄	SBI-09	119-155	132 -150	0.48	7
Xtxp012	CT ₂₂	SBI-04	143-215	169 -203	0.64	15
Xtxp015	TC ₁₆	SBI-05	197-273	199 -225	0.52	8
Xtxp021	AG ₁₈	SBI-04	145-227	167 -193	0.60	11
Xtxp040	GGA ₇	SBI-07	108-144	128 -137	0.32	3
Xtxp057	GT ₂₁	SBI-06	213-285	220 -254	0.56	8
Xtxp114	AGG ₈	SBI-03	196-245	212 -236	0.18	3
Xtxp136	GCA ₅	SBI-05	238-246	237 -243	0.06	3
Xtxp141	GA ₂₃	SBI-10	126-175	129 -185	0.72	12
Xtxp145	AG ₂₂	SBI-06	204-278	178 -248	0.81	16
Xtxp265	GAA ₁₉	SBI-06	163-246	178 -226	0.72	12
Xtxp273	TTG ₂₀	SBI-08	148-243	195 -237	0.53	8
Xtxp278	TTG ₁₂	SBI-07	225-319	242 -254	0.17	4
Xtxp320	AAG ₂₀	SBI-01	250-329	253 -289	0.48	8
Xtxp321	GT ₄ + AT ₆ + CT ₂₁	SBI-08	180-252	188 -218	0.60	12

ARLEQUIN program, version 3.11(Excoffier et al., 2005).

RESULTS

SSR polymorphism

The analysis of SSR products in this study showed fragment lengths similar to that reported in the earlier studies (Table 2). The 39 SSR markers produced a total of 261 alleles among the 200-sorghum accessions. The number of alleles per polymorphic locus ranged from two (mSbCIR246, Xcup061 and Xcup63) to 16 (Xtxp145) with mean 6.7. The Polymorphic Information Content (PIC) ranged from 0.06 (Xtxp136) to 0.81 (Xtxp145) with mean 0.45 (Table 2).

Extent and pattern of genetic diversity among accessions

Dice's similarity coefficient calculated based on 39 SSR markers ranged from 0.062 between Acc#225836 from Gamo Gofa and Acc # 226058 from Wello to 0.96 between Acc#242049 from Wello and Acc#69228 from Hararghe. These indicated that there was a fair amount of variation among the accessions studied. In addition to this, there were about 20 pairs of accessions, which showed dissimilarity value greater than 0.6.

Hierarchical clustering using UPGMA classified the accessions into three major groups (Figure 2). The first cluster (blue) consisted of 130 accessions, the second (green) 37 accessions, and the third (red) 30 accessions all mixed from different regions of collection. Moreover, the PCoA (Figure 3) revealed that there was no clear grouping of accessions according to the old agro-ecological classification, which bases altitude. Analysis of molecular variance (AMOVA) in this study, using collection region as a grouping criterion, 99.62% of the variation was accounted to among accessions while variation among regions of collection contributed only 0.38 % to the total variability observed (Table 3).

DISCUSSION

Ethiopia is endowed with rich natural resources including immense genetic diversity in cultivated plant species. *S. bicolor* is among the most diverse indigenous crop species in Ethiopia. The present study tried to uncover the extent and patterns of genetic diversity in sorghum gene bank accessions collected country wide.

Extent and patterns of genetic diversity

Wide range of variation was observed among accessions

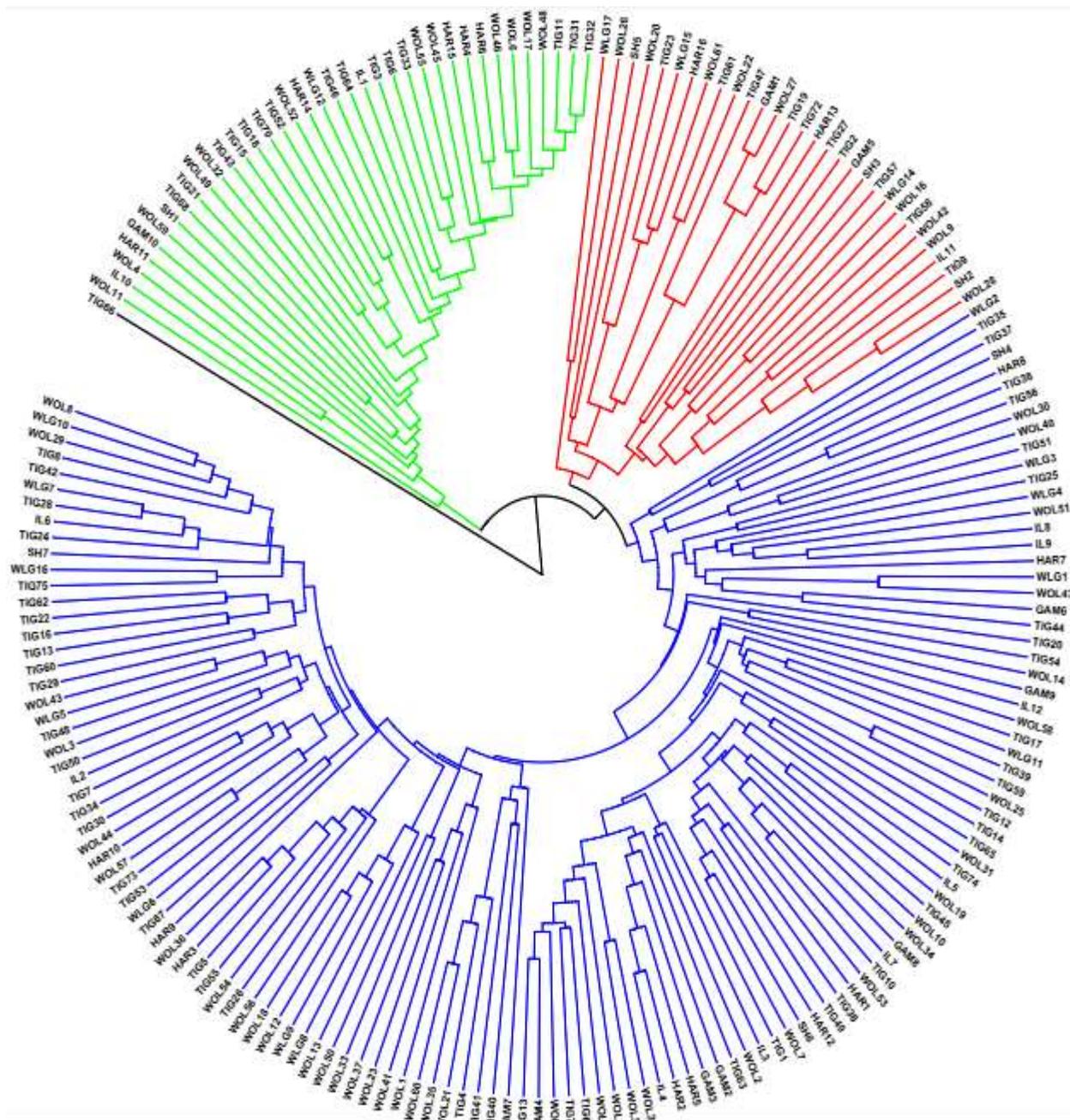


Figure 2. UPGMA analysis clustered the 200 sorghum accessions in to three broad groups, which are not related to geographical regions of origin.

for Dice's similarity coefficient calculated from 39 SSR markers. The fact that both hierarchical clustering and PCoA failed to reveal clear grouping of accessions based on either altitude or region of origin indicates these parameters are not good guides to follow for sorghum germplasm collection (Figures 2 and 3). Similar to this

study, lack of complete differentiation in Ethiopian and Eritrean sorghum accessions based on geographic origin was previously reported. Although variation in the growing environment among the different regions was substantial, genetic differences attributed to region of collection was not significant. This is because

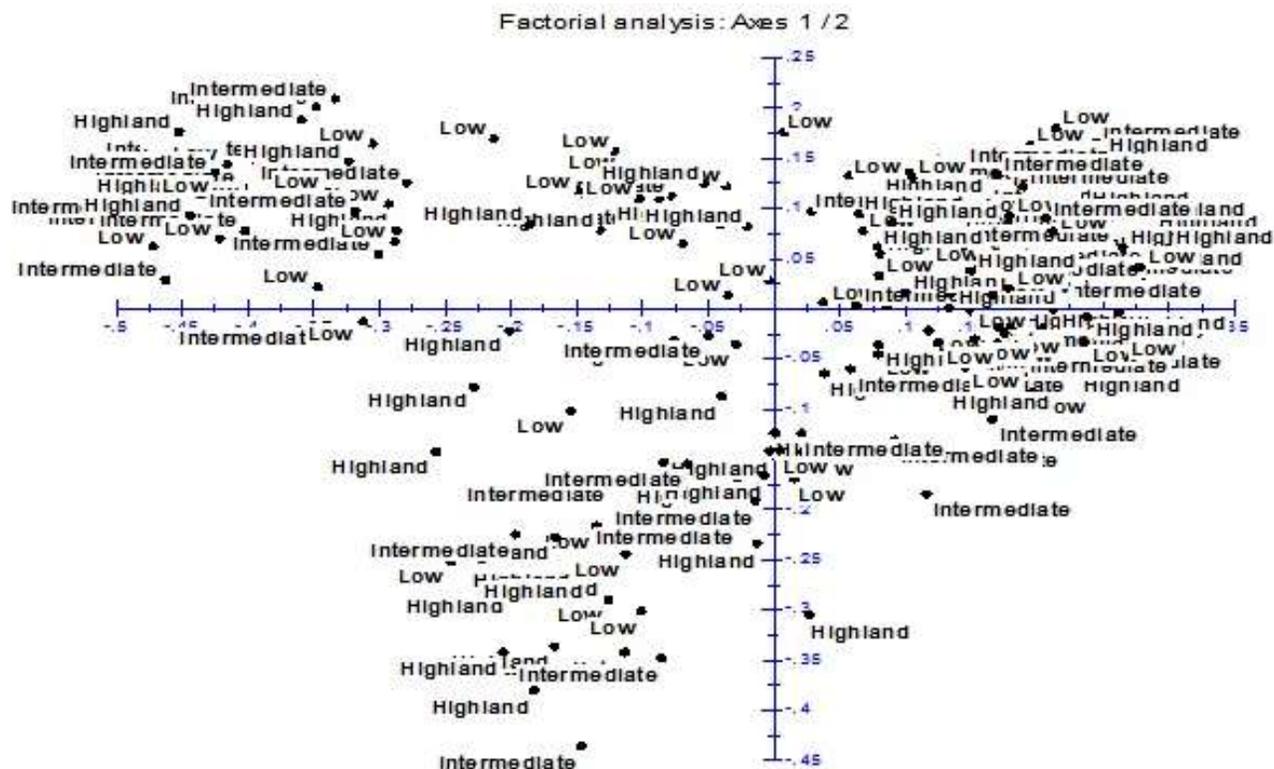


Figure 3. Principal coordinates analysis PCoA showing the clustering pattern of the 200 sorghum accessions based on altitudinal classes

Table 3. Analysis of molecular variance AMOVA describing the between and within region variability for SSR marker alleles

Source of variation	DF	Sum of squares	Variance components	Variation %	F _{ST}
Among regions	6	144.027	0.08431	0.38	
Within regions	189	4132.183	21.97970	99.62	0.00382
Total	195	4276.210	22.06400		

geographical regions in Ethiopia are not completely different in terms of climate and other soil variables, rather they share similar agro-ecologies. Adugna (2012) suggested that the latest agro-ecological zonation, which classified Ethiopia into 32 agro-ecologies out of which sorghum grows in 12 of them, were more informative to follow for sorghum germplasm collection than geographical regions of origin and the traditional classification using the four agro-ecologies (dry lowland, wet lowland, intermediate, and highland). Another reason could be because traits for adaptation to different conditions might have been the result of point mutations that are difficult to detect with the regular SSR marker assays.

The high variation among accessions observed in this study was in agreement with previous studies and confirms that sorghum is predominately inbreeder (Doggett, 1988). Ejeta and Grenier (2005) reported that genetic variation among sorghum accessions accounted for 86% of the total variation. Similarly, diversity study among 'Orange' accessions in the U.S. national collection using SSR markers showed that 90% of the total genetic variation was partitioned among accessions, while 10% of the variation was found within accessions also reported that a large proportion of genetic variation was observed within regions (88%) rather than between regions (12%) in wild sorghum from Ethiopia and Eritrea. Ng'uni et al. (2011)

also found that 82% of the total genetic variation was attributable to among accessions whereas 18% was within accessions. Adugna et al., (2013) studied *in situ* genetic diversity analysis in wild sorghum and reported that 58.8% of the variation was within populations. Similarly, Adugna (2014) reported 54.4% of the variation in cultivated sorghum landraces to be within populations.

Implications for future breeding and conservation efforts

Understanding of the extent and pattern of genetic variation can be useful for several reasons. Such information can be used to design effective *in situ* germplasm conservation and for setting germplasm collection mission as well as to estimate or predict the risk of genetic erosion in certain areas. From breeding standpoint, knowledge of the pattern of genetic variability is useful for defining heterotic groups in hybrid breeding and for relating the observed pattern with presence of certain economically important traits. Previously, the extent and pattern of genetic variability among the 200 Ethiopian sorghum [*Sorghum bicolor* (L.) Moench] germplasm accessions used in the present study were estimated using 26 morpho-agronomic traits at two locations and the result revealed the presence of wide range of genetic variability for all the 14 quantitative characters measured (Tesso et al., 2011).

The study has shown the presence of high genetic distance among some of the accessions. Moreover, UPGMA analysis clustered the 200 accessions into three large groups irrespective of the geographical regions of collection. Therefore, future crossing program can make use of the distant accessions identified in this analysis, and/or among accessions belonging to the three broad clusters. Based on the result of the current genetic diversity revealed by molecular genetic markers among the accessions, the following conclusions can be drawn: (1) the wide range of variability observed can be exploited in plant breeding to develop varieties with improved yield potential; (2) Although variation in the growing environment between the different regions was substantial, genetic differences for sorghum attributed to region of collection was not significant. Therefore, the old system of agroecological zonation may not be helpful in collection of germplasm. Rather, the latest agroecological zonation can serve better for this purpose.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Introduction of the exotic breeds and cross breeding of local chicken in Ethiopia and solution to genetic erosion: A review

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This study reviews the introduction of the exotic breeds and cross breeding of local chicken in Ethiopia, solution to genetic erosion and needs for conservation with aim of delivering in the short form and clear information for beneficiaries and to whom it may concern. Poultry production and its product consumption are progressively increasing globally. In Ethiopia, chicken production plays a great role as a primary supply of eggs and meat in rural and urban areas and as a source of income for smallholder farmers. The interest of the farmers to maximize poultry products is increasing. Therefore, the different parts of the farmers are practicing crossbreeding unsystematically. However, crossbreeding is not advised and recommended by government due to its impact on genetic diversity and losing of the important traits of local chickens; thus, policy of the government has prepared different strategies to improve livestock development. There is no good breeding scheme introduced so far to avoid such kind of impacts or else regulator laws to avoid uncontrolled breeding activities. Therefore, conservation of locally adapted indigenous livestock breeds has become an important objective in sustainable animal breeding, as these breeds represent a unique genetic resource. And even if the indigenous chickens are low in productive performance, it has its unique and important characteristics such as brooding, tolerance of the disease and harsh environment of tropics, and giving good product under management condition. Therefore, adjusting the proper breeding strategies in the country, especially upgrading the local chicken for higher production through selective breeding system rather than cross breeding with exotic breeds and conservation of the genetic resource is needed.

Key words: Conservation, cross breeding, exotic and local chickens breed, genetic erosion, Ethiopia

INTRODUCTION

The importance of native breeds of chicken for rural economy in developing and underdeveloped countries mostly in Asia and Africa is very high; since they are part

of balanced farming system that have vital roles in the rural households as a source of high quality animal protein and emergency cash income and play a

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significant role in the socio-cultural life of the rural community and woman empowerment (Mtileni et al., 2016).

However, indigenous chickens are better to adapt the harsh environment, disease tolerance and good brooders, but they are poor in the reproductive performance (Nigussie, 2011). Due to the lower productive performance of indigenous chicken, and having of the great interest on egg and meat production, the farmers trend in introducing, selecting and breeding exotic chickens are increasing from time to time (Matawork, 2016; Gebremariam et al., 2017). Many of the exotic breed's chickens are distributed by higher learning institutions, research organizations, the Ministry of Agriculture and certain Non-Governmental Organizations (NGOs) to the rural farmers and urban based on small-scale poultry producers. For instance, Tadesse et al. (2013) reported that the exotic chicken such as Isa Brown, Bovans Brown and Potchefstroom koekoek were distributed to smallholders of the Oromia region. Aman et al., (2017a) also reported that the private chicken farms (Ethio-chicken poultry farm) distributed 42 day old cockerels and pullets to the Wolaitta Zone in SNNPR State.

Different authors reported that there was no systematic breeding of the chicken in the developing countries. This may cause the inbreeding and loss of important (selective) traits of the indigenous chicken (Nega et al., 2016; Chen et al., 2016). As inbreeding usually leads to loss of vigor and fertility; therefore, the production recording has to be carried out for future development of chicken production (Muasya et al., 2013).

Hoffmann (2009) reported that the chicken genetic resources are probably the most endangered and under-conserved of all livestock species, with approximately 33% of the world's chicken breeds considered endangered. Therefore, conservation of locally adapted indigenous livestock breeds has become an important objective in sustainable animal breeding, as these breeds represent a unique genetic resource (Mtileni et al., 2016). Although animal genetic resources are playing a vital role in ensuring food security and maintaining genetic diversity, the way of conservation of animal genetic resources in developing countries is minor (Adebabay et al., 2016).

Even though, different authors such as CSA (2011), Tadesse et al. (2013), Geleta et al., (2013), Aman et al. (2017b), and Alem (2014) had reported that there was a good productive performance on the different breeds of crossbred and pure exotic chickens in the different parts of Ethiopia. Adaptability of the tropic environment, disease, low nutrition, housing and overall management strategies of the village farmers are challenging the chicken's performance of the exotic chickens in the country (Hailu et al., 2012; Dessie et al., 2013). Matawork (2016) also reported that exotic chickens are easily attacked by predators and poor adaptive ability to harsh

environment; they require high quantity and quality feed and clean environment to produce well.

However, the reports on native ecotypes in the tropics showed that their potential for egg production and growth is very low under smallholder farmer's management conditions. A level of production increases significantly under the improved feeding, housing and healthcare conditions (Dessie et al., 2011). Upgradation of the native breeds of chickens through different breeding techniques helps to increase the productivity and also their conservation in their natural habitat as the rural people will be very happy to rear them for their adaptability to harsh environment (Padhi, 2016).

Since the need of the farmers for maximizing the income through meat and eggs, the pressure of the importation of the exotic animal and the shift of the production system into the commercialized once and breeding schemes are making the genetic erosion on the local (indigenous) chicken breeds of the country.

Therefore, based on the aforementioned background, this review was conducted within objectives to review the introduction of the exotic breeds and cross-breeding of local chicken in Ethiopia, solution to the genetic erosion and needs for conservation, breeding system and performance of the chicken in the country and also the way of genetic conservation of local chickens.

ETHIOPIAN POULTRY POPULATION AND DISTRIBUTION

Poultry include all domestic birds kept for the purpose of human food production (meat and eggs). However, the term "poultry" production under Ethiopian context is synonymous with chicken production (CSA, 2012). According to CSA (2015), major proportion of chickens reared in the country is of indigenous types with a minuscule number accounting for the crossbreds and exotic chickens. CSA (2016) also reported that the total poultry population in Ethiopia is estimated to be about 60.5 million, of which 94.3, 3.2 and 2.5% were reported to be indigenous, cross and exotic, respectively in backyard, small-scale, and large-scale commercial production systems. But Gebremariam et al. (2017) reported that the trend of the rearing of the exotic chicken in the study areas was increasing.

It is believed that the improved breeds of chickens were introduced to Ethiopia by the missionaries sometimes in the mid decades of the last century (Meseret, 2010). Four breeds of exotic chicken (Rhode Island Red, Australop, New Hampshire and White Leghorns) were imported to Jimma and Haromaya in 1953 and 1956, respectively under USAID project (Solomon, 2007). Matawork (2016) also reported that due to the low performance of indigenous chicken, the introductions of highly productive breeds of exotic chicken were done in urban, peri-urban and rural areas in the country.

POULTRY PRODUCTION SYSTEMS IN ETHIOPIA

The poultry sector in Ethiopia can be categorized into three major production systems based on some selected parameters such as genotype, flock size, housing, feed, health, technology, and bio-security. Thus, poultry production can be classified as backyard poultry production system, small scale poultry production system and commercial poultry production system (Bush, 2006; FAO, 2007).

BREEDING SYSTEMS OF THE CHICKEN IN ETHIOPIA

Selective breeding for the improvement of the productivity in indigenous Horro chicken in Ethiopia was started in 2008 (Dana et al., 2011).

However, crossbreeding is not advised and recommended by agricultural extension due to the impact on genetic diversity and losing the important traits of local chickens (Gebremariam et al., 2017). There is no good breeding scheme introduced so far to avoid such kind of impacts or else regulate lows to avoid uncontrolled breeding activities (Southern zone of Tigray (SZT), 2014). According to different studies reported by Addisu et al., (2013), Nega et al. (2016), Alemayehu (2017), and Gebremariam et al. (2017), there is uncontrolled breeding system of the chicken under the village chicken production system in the different parts of the country. Nigussie (2011) also reported that there is no systematic mating in any regions of Ethiopia.

Crossbreeding between commercial cocks and indigenous hens may provide a way to produce productive dual-purpose chickens, and that can cope with harsh environments. For instance, the Rhode Island Red (RIR) has been the most common commercial line used to obtain dual-purpose chickens by crossing with indigenous birds (Khawaja et al., 2012; Bekele et al., 2010). However, since crossbred chickens are usually heavier than indigenous chickens (Desalew, 2012), there are some potential disadvantages of such crossbreds. For example, they require higher amount of feed than indigenous chickens and therefore, crossbred chickens may suffer from poor nutrition and diseases in an environment where feed is limited (Matawork, 2016). Therefore, it was suggested that implementing a selective breeding program to improve indigenous chickens is an alternative for crossbreeding to increase productivity (Wondmeneh, 2015).

PRODUCTIVITY OF CROSSBRED AND EXOTIC CHICKENS IN ETHIOPIA REARED UNDER FREE RANGE SYSTEM

There are also farmers who keep modern breeds in small flock sizes and use relatively improved management (Besbes, 2009). Even if the indigenous chickens are

better to adapt the harsh environment, disease tolerance and good brooders, they are poor in the reproductive performance. Therefore, to improve the performance of the local chickens, the exotic chickens such as white and brown Leghorns, Rhode Island Red, New Hampshire, Cornish, Australop Light Sussex, etc., were imported to the country and crossed with local chicken (Nigussie, 2011). Since then the higher learning institutions, research organizations, the Ministry of Agriculture and certain Non-Governmental Organizations (NGOs) have distributed many exotic breeds of chicken to rural farmers and urban-based small-scale poultry producers (Pagani and Wossene, 2008). For instance, Tadesse et al., (2013) reported that exotic chicken such as Isa Brown, Bovans Brown and Potchefstroom koekoek were distributed to smallholders of the Oromia region. Aman et al., (2017a) also reported that the cockerels and pullets (42-day old chicken) were distributed by the private chicken farms (Ethio-chicken poultry farm) to the Wolaitta Zone in SNNPR State and they also reported that the egg production and overall productive performances of exotic (Sasso) chickens were better than the indigenous chickens. Aklilu et al., (2013) also reported that the Potchefstroom koekoek breeds chicken were reared and showed good performance better than indigenous chickens under village production system of Afar region. Tadesse et al., (2013) reported that the exotic chicken such as Isa Brown, Bovans Brown and Potchefstroom koekoek showed good productive performance under farmers' management conditions in Oromia region. Dirsha (2009) also reported Rhode Island Red (RIR) chickens showed better production performance in Cheha Woreda, Ethiopia. Haftu, (2016) also concluded that exotic breed and cross breed chickens can produce a large number of eggs in the presence of adequate amount of feed.

PERFORMANCE OF INDIGENOUS CHICKEN UNDER IMPROVED MANAGEMENT CONDITIONS

Reports on native ecotypes in the tropics showed that their potential for egg production and growth is very low under smallholder farmer's management conditions. However, under improved feeding, housing, and healthcare conditions, levels of production increased significantly (Desie et al., 2011). The mean body weight gain of local chickens of Ethiopia on station management was higher than traditional management (Wondmeneh, 2015). Wondmeneh, (2015) also reported that egg production of the local chickens was increased after 6 generations of selection, and the analysis revealed positive genetic changes over generations. An Iranian native chicken body weight and egg weight was also increased through selection and breeding program (Bahmanimehr, 2012). Wondmeneh, (2015) reported that the level of performance of improved chickens were lower than that of commercial chickens or crossbreds. And the

same author reported that this difference in performance is expected to decrease with further generations of selection in the Improved Horro.

IMPORTANCE OF GENETIC RESOURCE AND ITS CONSERVATION

It is so clear that the importance of animal genetic resources is judged most often from the direct value of livestock breeds and conservation. The breed improvement programs solely depend on the direct values of animal genetic resources (Adebabay et al., 2016). Naqvi (2007) reported that the animal genetic resources are also very essential in research and training activities like research in immunology, nutrition, reproduction, genetics and adaptation to climatic and other environmental changes. Okeno et al., (2012) suggested that breeding program targeting improvement of indigenous chicken should focus on within breed selection rather than crossbreeding with commercial chicken breeds, this would help to maintain the indigenous chicken unique attributes which are appreciated by producers and avoid genetic erosion and dilution and contribute to their conservation. The report published by Padhi (2016) suggested that upgrading the native chicken by different breeding techniques and conservation in their habitat is important. The same author reported that the rural people would be happy rearing the upgraded native chickens for they can adapt to too harsh environment beside improve productivity.

CAUSES FOR GENETIC EROSION OF CHICKEN IN ETHIOPIA

Pressure to adopt improved animal

Although IBC (2005) reported that the threats for indigenous chicken population could be pictured as a pool of gene under pressure mainly by the replacement with the exotic breeds (crossbred). The crossbreeding and imports of exotic breeds are practical options for specialized and improved family poultry systems in all zones of the country (LMP, 2015). FAO (2007) also reported that the main cause of genetic erosion in developing countries is attributed to the fact that farmers have a strong pressure to switch to commercialized livestock production and breeding schemes, because of agricultural policies promoting rapid solutions to ensure food security or meeting the soaring demand for food (LML, 2015).

Paradigm shifts in the production system

In addition, due to the shift of production system from backyard to small-scale and large-commercialized

system, the production system will become more intensive; the large input and highly producing exotic chicken will be needed. In effect, the paradigm shift in production system has led to increase the use of exotic genetic resources, often at the expense of indigenous livestock breeds (FAO, 2010). Gebremariam et al. (2017) from Northern Ethiopia also reported that there were higher number of the exotic chickens in the study area and the trend of the rearing of the exotic chicken was increasing due to the fact that the farmers get interest on egg and meat production.

Less contribution of extension and livestock development agents

Gebremariam et al. (2017) suggested that the less number of the local breeds and comparative low contribution of crossbreds might be due to the impact of extension agents and disseminators training on negative effects of uncontrolled crossbreeding as a root cause of genetic erosion. LMP (2015) reported the absence of the accessible information for decision-makers, no approved livestock breeding policies, regulations, and strategies for the breeding of chickens. Adababay et al. (2016) also suggested that there should be strategy for capacity building among farmers and local communities, through education and training, awareness raising, information sharing and the dissemination of case studies. The same author also suggested that a strategy for controlling imports of exotic breeds and provision of information on the potential consequences and priority for indigenous animal breeds conservation is also needed.

CURRENT STRATEGIES AND STATUS OF ETHIOPIAN GOVERNMENT ON IMPROVEMENT AND CONSERVATION OF NATIVE BREEDS OF CHICKEN

According to the LMP (2015) report, the government would focus on local breed improvement through both within-breed selections of indigenous breeds and crossbreeding with exotics, giving due attention to biosecurity and genetic conservation. As many breeds and species in Ethiopia are not well characterized, Niguse (2011) characterized five breeds and studied the local breed production and reproduction, as well as other characteristics and he recommended that it has improved the performance through organization of within breed selection procedures. The government has also set the plan in the draft document on crossbreeding, the importation of exotic breeds (both pure and crossbreds of exotics) and genetic materials, the establishment of multiplication centers, the development of national livestock recording schemes, breed quality, certification and control systems, and genetic improvement research (LMP, 2015).

However, the policy of the government has different

strategies to improve livestock development such as identify breeding objectives for each species, undertake structured and continuous within-breed selection for best-performing indigenous breeds and distribute and/or cross them with other indigenous breeds, implement community-based local breed improvement schemes, give training to farmers on genetic improvement activities and better animal husbandry (LMP, 2015). There is also the gap of the linkage between the strategies and the work done practically under farmer level. Uncontrolled breeding of the chicken occurring in the different parts of the country (Addisu et al., 2013; Nega et al., 2016; Alemayehu, 2017; Gebremariam et al., 2017), less contribution of the extension agents by giving training to farmers (Gebremariam et al., 2017), the chicken suppliers are not providing either technical or any other support to exotic poultry farm owners (Desie et al., 2013).

CHALLENGES TO MEET THE TARGET OF THE STRATEGIES TO PROMOTE GENETIC IMPROVEMENT

However, some genetic improvement success has been reported in the poultry sector and the draft breeding policy focuses on local breed improvement (LMP, 2015). Much of the work is needed to improve the indigenous chicken and many studies suggested lack of systematic breeding strategies under farmers level in different parts of the country (Addisu et al., 2013; Nega et al., 2016; Alemayehu, 2017; Gebremariam et al., 2017).

Therefore, the LMP (2015) reported that there are many challenges in Ethiopian livestock genetic improvements; for instance, challenges listed in the country are lack of genetically-improved indigenous animals and genetic materials, no approved livestock breeding policies, regulations and strategies, absence of accessible information for decision-makers and finally, lack of a national database on genetic improvement and progress.

EFFECTIVE AND SUITABLE WAY OF GENETIC CONSERVATION AND IMPROVEMENT OF THE INDIGENOUS CHICKEN

Selective breeding

Nigussie (2011) reported that the selection program in Horro chicken of Ethiopia is the strong association between body weight at 16 weeks and egg production from 21 to 28 weeks. Moreover, the low to moderate heritability estimates for different traits indicates that the performance of Horro chicken can be improved through suitable selection program. An Iranian native population selected on the basis of breeding value recorded moderate to high heritability estimates and higher

heritability estimates for body weight; this suggests that improving the body weight and egg weight through selection and breeding program can be achieved (Bahmanimehr, 2012). Haunshi et al., (2012) reported moderate to high heritability estimates in Aseel (0.22 to 0.49) and Kadaknath (0.22 to 0.37) for juvenile body weight and shank length which indicates as a scope for further improvement through selection.

One of the most important positive characters of native chicken is their hardiness, which is the ability to tolerate the harsh environmental conditions and poor husbandry practices without much loss in production (Padhi, 2016). Therefore, the breeding program to improve the performance of indigenous breeds of chicken through selection is of great help to the farmers in the rural areas to improve their earning from indigenous birds (Menge et al., 2005). Padhi (2016) reported that low production performance of native breeds of chickens may be improved through improvement in husbandry practices, better healthcare, and supplementary feeds during lean season and also through selection.

CONCLUSION

Due to low performance of indigenous chicken, the government of Ethiopia has made attempt to introduce different exotic poultry breeds to smallholder farming systems of Ethiopia. Despite management problems involved in rearing poultry, the exotic breed chickens are appreciated for their more egg production, but sensitive to disease, predators and feed shortage in Ethiopia. The farmers' interests to maximize their incomes from chickens are also increasing. Therefore, most of the farmers are practicing crossbreeding (unsystematically). However, the policy of the government prepared different strategies to improve livestock development; there is also the gap of the linkage between strategies and the work done on practically under farmer level; for instance, uncontrolled breeding of the chicken occurring in the different parts of the country. Due to this reason, the indigenous chicken breed genetic is losing its value and economically important traits.

RECOMMENDATION

Even if the indigenous chickens are low in productive performance, it has its unique and important characteristics such as brooding, tolerance of the disease and harsh environment of tropics, giving a good product within low management. Therefore, the following recommendations are forwarded to whom in may concern.

(1) Adjusting the proper breeding strategies in the country especially upgrading the local chicken for higher production through selective breeding system rather than

cross breeding with exotic breeds.

2) Indiscriminate random mating among the indigenous (chicken and unplanned crossbreeding with exotic breeds should be minimized.

(3) Awareness among livestock keepers about the potential roles of animal genetic resources in the country in terms of adaptation of their natural environment and training the people for conservation of indigenous breed is essential.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Effect of methanolic extract of *Dennettia tripetala* (pepper fruit) on biomarkers of oxidative stress and lipid peroxidation in type 2 diabetic male wistar rats

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Oxidative stress resulting from chronic hyperglycemia in diabetes is due to high production of reactive oxygen species and/or a decrease in the antioxidant defense system activity. The study was designed to investigate the ameliorative effect of methanolic extract of *Dennettia tripetala* (DT) on oxidative stress and blood glucose level in type II diabetic male Wistar rats. Type II diabetes was induced by a single intraperitoneal injection of streptozotocin (40 mg/kg) after 2 weeks of 10% fructose diet. Twenty-five (25) rats were randomly divided into five groups, namely: 1 (normal control), 2 (negative control), 3 (10 mg/kg of glibenclamide), 4 (100 mg/kg of DT extract) and 5 (200 mg/kg of DT extract). The administration of the extract caused a significant decrease in blood glucose levels in all treatment groups. A significant ($P<0.01$) decrease in malonaldehyde (MDA) activity was observed in the group treated with 100 and 200 mg/kg of DT extract compared to diabetic control. Catalase (CAT) activity showed a significant ($P<0.05$) increase in the group treated with 200 mg/kg of DT extract compared to the normal control. However, the extract did not affect GSH-Px, SOD and CAT activities. The findings suggest ameliorative effect of DT extract in diabetic Wistar rats.

Key words: *Dennettia tripetala*, diabetes mellitus, oxidative stress, lipid peroxidation, Wistar rats.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease in which there is a high blood sugar level over a prolonged period (WHO, 2014), due to the inability of one's body to

properly use the energy from the food they eat. This is caused by inherited and/or acquired deficiency in production of insulin by the pancreas or by the

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ineffectiveness of the insulin produced. Nigeria has a population of 186 million people and according to International Diabetic Foundation (IDF); 2 million of this population is believed to be known diabetics while a greater percentage are still undiagnosed. According to the World Health Organization (2014), the number of people with diabetes in Africa has jumped from 4 million in 1980 to 25 million in 2014. The disease was responsible for more than 320,000 deaths in 2015.

Oxidative stress is the outcome of an imbalance between the production and neutralization of reactive oxygen and nitrogen species (RONS) such that the antioxidant capacity of the cell is overwhelmed (Chikezie et al., 2018). Oxidative stress is a common mediator in pathogenicity of established risk factors, diabetes mellitus being one of them (Ho et al., 2013). The primary causative factor of oxidative stress in diabetes is the hyperglycemia (Chikezie et al., 2018).

Dennettia tripetala hereafter referred to as DT, is also known as pepper fruit, a member of Annonaceae family (Okwu, 2004). It is widely grown in the rain forest zones of Nigeria and some parts of West Africa. It is known in Nigeria by the following names: Ako (Edo), Mmimi (Igbo), and Ata Igbere (Yoruba). The fruits are green when developing but start to turn red with ripening. The fruits possess a very strong characteristic smell while the fruits and seeds are edible and are consumed because of the spicy nature. This highly nutritious fruit is rich in fatty acids, carbohydrates, proteins, calcium, potassium, magnesium, phosphorus, niacin, riboflavin, thiamine and vitamin A, C and E (Isegholi, 2015). This plant possesses phytochemical that have been shown to elicit antimicrobial, insecticidal, analgesic, and anti-inflammatory properties (Enwere, 1998). Diabetes is a serious medical problem worldwide, and due to the fact that most of the common menu is being restricted because of the carbohydrate content, a lot of diabetic patients are in search of healthy and cost-effective diets and better treatment strategy (WHO, 2014).

D. tripetala is used in traditional medicine as a remedy for cough, fever, toothache, diarrhea, diabetes, and nausea in pregnant women (Ejечи and Akpomedaye, 2005). However, there is paucity of information on the antidiabetic effect and specifically its effect on biomarkers of oxidative stress in diabetes. There is need to investigate the anti-hyperglycemic effect of *D. tripetala* in order to explore the possibility of using it as a non-pharmacological means of treating diabetes by low income earners of the tropical areas, especially in the rural and under-developed countries. This present study is also designed to investigate the effects of *D. tripetala* on biomarkers of oxidative stress in order to ascertain its ability to reduce the complications of diabetes, which are the major causes of mortality of the disease. This study was aimed at assessing the effect of methanolic extract of *D. tripetala* on biomarkers of oxidative stress and lipid peroxidation in type 2 diabetic male Wistar rats.

MATERIALS AND METHODS

Plant purchase, identification and authentication

D. tripetala fruits were purchased from New Market in Enugu metropolis, Enugu State. A sample of the fruit was identified and authenticated at the herbarium section of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. A voucher specimen was deposited in the herbarium for further reference with the number: UNH no 8c.

Extraction process

The method of Onyeso et al. (2016) was adopted and modified. *D. tripetala* fruits were properly de-stoned and pulverized using a mechanical milling machine. 1000 g of pulverized seeds was macerated in 5000 ml of a mixture of 80% methanol and 20% distilled water. This was followed by vigorous shaking at 1 h intervals for 48 h. After which, it was filtered using a clean sieve with tiny holes and finally filtered using No. 1 Whatman filter paper and measuring cylinders. The filtrates were poured into clean beakers and the solvents evaporated using moist air oven by Drawell Scientific Limited China (Model: DGT-G25). The extracts after evaporation were weighed and a total of 36 g of the pure extract was kept in a refrigerator at -2°C till ready for use.

Experimental animals

A total of forty (40) Wistar rats were purchased from the Animal House Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State. The rats weighed between 110 and 160 g and were housed in stainless steel and well ventilated cages under controlled environmental conditions (12 h light/dark cycle) at the animal house unit of the Department of Anatomy, Faculty of Basic Medical Science, University of Nigeria, Enugu campus, Enugu.

Ethical clearance

Ethical clearance for this study was obtained from the Research Ethical Committee, College of Medicine, University of Nigeria, Enugu with protocol number 037/12/2017.

Preparation and administration of streptozotocin solution for the induction of diabetes

The solution of streptozotocin was prepared according to the method of Anioke et al. (2017). A total of 250 mg of powdered Streptozotocin purchased from Bridge Biotech, Ilorin (Santa Cruz Biotechnology, USA) was weighed using an electronic weighing balance and dissolved in 100 ml mixture of 0.1 M sodium citrate buffer (a mixture of 46.5 ml citric acid and 3.5 ml of sodium citrate solution) which served as the vehicle to get the appropriate stock concentration of 30 mg/ml. This was administered to the rats after overnight fast (8-12 h). The rats were induced with diabetes individually by single intraperitoneally administration of the dissolved streptozotocin powder at a dose of 40 mg/kg body weight. Using the formula stated as follows:

$$\text{Volume} = \frac{\text{dose} \left(\frac{\text{mg}}{\text{kg}} \right) \times \text{weight} (\text{kg})}{\text{stock concentration} \left(\frac{\text{mg}}{\text{ml}} \right)}$$

Preparation of 0.1 M citric acid and sodium citrate solution

Approximately 2.87 g of powdered citric acid ($C_6H_5O_7NO_3 \cdot 2H_2O$) with molecular weight of 287 g/mol was weighed and dissolved in 100 ml of distilled water. Also 100 ml of 0.1 M solution of sodium citrate ($C_3H_4(OH)(COONa)_3 \cdot 2H_2O$) was prepared following the same method. Then 44.5 ml of citric acid was mixed with 55.5 ml of sodium citrate and the mixture standardized with citric acid to a pH of 4.5. The preparation of 0.1 M solutions was done using the method of Ojokuku (2002). The streptozotocin dissolved in citrate buffer (pH 4.5) after an overnight fast was to induce partial pancreatic β -cell destruction. Two days (48 h) after induction, diabetes was confirmed with a fasting blood glucose level of ≥ 200 mg/dl, using the ACCU-ANSWER Glucometer (ZH-G01) produced by Guangxi-China (Mainland) using a whole blood obtained from the caudal vein of the animals after a little incision with scissors.

Experimental design

Group 1 = Normal control rats; received feed and water *ad libitum*
 Group 2 = Diabetic untreated group (negative control)
 Group 3 = Diabetes + treatment with 10 mg/kg Glibenclamide orally only (positive control)
 Group 4 = Diabetes + treatment with 100 mg/kg methanolic extract of *D. tripetala* only
 Group 5 = Diabetes + treatment with 200 mg/kg methanolic extract of *D. tripetala* only

Acute toxicity studies

The median lethal dose (LD_{50}) was determined by method of Lorke (1983) using twelve rats weighing between 100 and 190 g. In the first phase, four rats were divided into two groups of two rats each and they were treated with the extract of the fruit at 1600 and 2000 mg/kg body weight intraperitoneally, and then observed for 24 h for sign of toxicity. In the second phase eight rats were then divided into four groups of two rats each and also treated with extract of the fruit at doses 3000, 4000, 5000 and 6000 mg/kg of body weight intraperitoneally. The median lethal doses were then calculated as the square root of the highest dose that gave no mortality ($\sqrt{D_0}$) multiply by the lowest dose that gave mortality (D_{100}). $LD_{50} = \sqrt{D_0} \times D_{100}$

Administration of extract

D. tripetala extract was administered orally to the ten rats in groups 4 and 5 for 4 weeks giving a total of twenty-eight (28) days, with the use of an oral gastric gavage and 5 ml syringe. Administration was done in the evenings after which the rats were fed. Group 3, the positive control group, was given the standard diabetes drug for the same duration. *D. tripetala* extract was administered orally to the ten rats in groups 4 and 5 for 4 weeks giving a total of twenty-eight (28) days, with the use of an oral gastric gavage and two 5 ml syringe.

Preparation of urethane for anaesthesia

At the end of the treatment period, 1.25 g of granulated urethane purchased from Sigma Aldrich (Steinhen, Switzerland) was weighed using an electronic weighing balance and dissolved in approximately 5 ml of distilled water and administered intraperitoneally at a dose range of 500 mg/kg. Blood was collected via cardiac puncture using a sterile hypodermic syringe and introduced into a sterile sample and the blood allowed to clot followed by centrifugation and serum was collected using an automatic pipette for the analysis of the

biomarkers of oxidative stress as stated.

Sample biochemical analysis

Malondialdehyde (MDA) activity, a measure of lipid peroxidation, was analyzed using the method described previously (Ohkawa et al., 1979). The supernatant (0.2 ml) of homogenate was pipetted out in a test tube, followed by addition of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 30% acetic acid (pH 3.5), 1.5 ml of 0.8% of thiobarbituric acid and the volume was made up to 4 ml with distilled water. The test tubes were incubated for 1 h at 95°C, then cooled and 1 ml of distilled water was added followed by addition of 5 ml of n-butanol-pyridine mixture (15:1 v/v). The tubes were centrifuged at 4000 g for 10 min. The absorbance of developed pink color was measured spectrophotometrically (DU 640B spectrophotometer, Beckman Coulter Inc., CA, USA) at 532 nm. MDA was expressed as mmol/ml.

Glutathione peroxidase (GSH-Px) erythrocyte activity was measured according to Paglia and Valentine (1967). In the presence of glutathione reductase and NADPH the oxidized glutathione is immediately converted into the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. GSH-Px was expressed in unit per milligram protein (U/mg protein).

Superoxide dismutase (SOD) activity was measured according to the method described by Fridovich (1983). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with *p*-iodonitrotetrazolium violet (INT) to form a red formazan dye which was measured at 505 nm. The assay medium consisted of the 0.01 M phosphate buffer; CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2; solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 U/L xanthine oxidase. SOD activity was expressed as unit per milligram protein (U/mg protein).

Catalase (CAT) activity was measured following the method described previously (Beutler, 1984). The activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of hydrogen peroxide (H_2O_2) in phosphate buffer, pH 7.0 and requisite volume of serum sample. The molar coefficient of extinction (MCE) of 43.6 mcm^{-1} was used to determine the catalase activity. The assay medium consisted of 1 M Tris-HCl-5 mM Na_2 EDTA buffer solution (pH 8.0), 1.0 M phosphate buffer solution (pH 7.0) and 10 mM H_2O_2 . CAT activity was expressed as unit per min per milligram protein (U/min/mg protein).

Statistical analysis

The results obtained from this study were analyzed using statistical package for social science (SPSS version 21.0 for windows IBM Corporation, Armonk NY). Analysis of variance (ANOVA) was used to compare means and subjected to Tukey HSD for post Hoc for multiple comparisons, and values were considered statistically significant at $p < 0.05$ or $P < 0.01$. All results are presented as mean \pm standard error of mean (SEM).

RESULTS

The LD_{50} for *D. tripetala* seed extract was 5785 mg/kg and this evoked paralysis in rats for 4 days coupled with discharge from the eyes and eventual death.

Table 1 shows that in the treatment group 3, there was a significantly decreased ($P \leq 0.05$) blood glucose level

Table 1. Mean of the blood glucose levels (mg/dl) of all the experimental groups treated with DT extract.

Group	Week 0	Week 1	Week 2	Week 3	Week 4
1	123.3±2.55	114.0±1.67	124.5±2.43	113.0±2.65	113.3±2.44
2	468.0±33.94*	558.0±18.83*	441.5±32.26*	304.0±6.67	297.5±28.81
3	453.0±41.01*	136.5±1.58 ^β	130.8±4.12 ^β	114.8±3.03 ^β	95.0±1.95 ^β
4	389.8±36.02*	353.0±47.45	238.5±24.53 ^β	182.0±14.59	122.8±2.82 ^β
5	377.3±40.34*	312.8±44.26	205.3±14.30 ^β	153.0±9.74	141.8±3.74

Values were expressed as mean ± SEM. *Significant difference at P<0.05 compared to group 1 and ^βIndicates a significant difference at P<0.01. compared to group 2.

Table 2. Percentage changes in blood glucose levels between initial and weeks 2 and 4 respectively.

Group	%Δ between initial and week 2	%Δ between initial and week 4
1	0.81±0.12	-8.11±0.1
2	-5.66±1.68	-36.43±5.13* ^β
3	-71.13±6.9* ^β	-79.03±8.4* ^β
4	-38.81±2.9* ^β	-68.5±5.2* ^β
5	-45.59±3.2* ^β	-62.42±4.6* ^β

Values were expressed as mean ± SEM. *Significant difference at P<0.05 compared to group 1 and ^βSignificant difference at P<0.01 compared to group 2.

after the first week although groups 4 and 5 had a decreased blood glucose that was not significantly different (P>0.05). There was a significant decrease in blood glucose levels in group 3 in respect to group 2 in week 1. In the second week of this experiment, there was further significant decrease in groups 3, 4 and 5 still in respect to group 2. Table 2 shows the percentage changes in blood glucose levels between initial and weeks 2 and 4, respectively. Percentage reduction in blood glucose level was the highest in group 3 at both weeks 2 and 4, there was still a significant decrease in groups 4 and 5 for both weeks and in group 2 at week 4.

The malondialdehyde (MDA) levels of the experimental animals were only significantly different (P≤0.05) in group 3, which were lower than that of group 1. The difference in the groups was only significant at P≤0.01; there was a decrease in group 2 as compared to group 1, in group 4 as compared to groups 1 and 2 and also in group 5 as compared to group 2. There was not any significant difference (P>0.05) in the levels of GSH-Px, although there was a decrease in groups 2, 3 and 4 when compared with group 1 and an increase in group 5 as compared to group 1. The superoxide dismutase levels (SOD) levels were significantly increased (P<0.05) in group 3 as compared to group 2, although there was an increase in groups 3 and 4 when compared with group 2). The differences in the catalase activity (CAT) levels of the experimental animals were only significant (P≤0.05) in group 5, which was significantly increased as compared to group 1. Although there was an increase in the other groups (2, 3 and 4) when compared with group 1.

In Table 3, the diabetic negative control rats showed a significantly (P≤0.01) elevated levels of MDA compared with normal control rats. The diabetic positive control group that received glibenclamide showed a significant (P≤0.05) decrease in MDA levels compared to diabetic control group. The extract treated diabetic groups showed a significantly (P≤0.01) decreased lipid peroxidation as compared to the normal and diabetic control groups, 100 mg/kg of the extract had the lowest value for MDA as opposed to the 200mg/kg of *D. tripetala* extract which had a more increased MDA level. The changes in the GSH-Px levels were not significant (P>0.05), although the glibenclamide treated group had a decreased GSH-Px levels as compared to the diabetic negative and normal control group. The diabetic negative control group had a lower GSH-Px level as compared to normal control. The extract groups had increased level of GSH-Px as compared to diabetic controls and this increase was dose dependent with the values of 200 mg/kg higher than 100 mg/kg.

Table 4 shows a gradual increase in weight of the experimental animals in the various groups with the progression of the work, although this gradual weight gain was not significant. Group 1 had the highest increase in body weight by week 2 and 4 and with group 2 having the lowest at week 2 and group 4 the lowest at week 4. There was a significant increase in weight of animals in group 1 as compared to 2 in week 2, and a significant increase in percentage change in body weight of animals in groups 3 and 4. Overall, a significant (P<0.05) decrease in weight gain was observed in groups 2, 4 and 5 compared to group 1.

Table 3. Biomarkers of oxidative stress and lipid peroxidation in experimental animals.

Group	MDA (10^{-3}) nmol/mg protein	GSH-Px U/mg protein	SOD U/mg protein	CAT U/min/mg protein
1	8.5±1.01	3.61±0.53	4.17±0.42	0.81±0.14
2	12.8±0.5**	3.07±0.28	2.50±0.09	0.94±0.19
3	6.4±0.65*	2.81±0.38	5.05 ±1.12 ^β	0.87±0.03
4	3.3±1.35 ** ^{ββ}	3.28±0.46	3.87 ±0.36	1.07±0.03
5	8.38±0.79 ^{ββ}	3.72±0.48	4.14±0.48	1.46±0.42*

Values are expressed as Mean ± SEM, *P≤0.05 were considered significant to group 1, **P ≤ 0.01 compared to group 1. ^βP≤0.05 compared to group 2, ^{ββ}P≤0.01 compared to group 2.

Table 4. The basal and weekly body weights (Grams) of experimental animals.

Group	Week 0	Week 1	Week 2	Week 3	Week 4	Weight gain (Week4-Week1)
1	124.5±2.43	149.8±7.67	174.8±3.52	183.6±8.51	188.1±4.49	63.6±2.06
2	121.7±2.48	124.3±8.44	127.0±2.83	138.7±3.50	140.8±7.46	19.1±5.16*
3	145.9±2.88	163.2±5.88	169.6±6.91	188.2±5.87	202.0±6.81	56.1±3.93
4	153.6±5.17	164.2±8.15	165.6±4.97	173.7±5.26	175.7±6.67	22.1±1.50*
5	147.8±2.41	162.3±2.14	161.2±2.08	166.3±1.77*	177.2±2.69	29.4±0.28*

Values are expressed as Mean± SEM; *P≤0.05 were considered significant to 1, **P ≤ 0.01 compared to 1

DISCUSSION

Diabetes is associated with a number of metabolic alterations and principal among these is hyperglycemia. Known secondary consequences of hyperglycemia such as cellular damage, increased extra cellular matrix production and vascular dysfunction have all been implicated in the pathogenesis of vascular disease type II diabetes (Dalle-Donne et al., 2006). Free radicals and oxidative stress may act as a common pathway to diabetes itself, as well as to its complications (Wolff, 1993). This study was conducted to evaluate the effect of pepper fruit on blood glucose level, MDA, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) in type two diabetes. From the results obtained, the blood glucose level which was used as an index of metabolic control was significantly increased in the diabetic groups after induction, which is one of the causes for increased production of free radicals by direct Amadori reaction (Wolff, 1993). Treatment with medium-dose of *D. tripetala* extract (100mg/kg body weight) and high-dose of DT extract (200 mg/kg body weight) produced an increased percentage reduction in blood glucose level comparable to glibenclamide. The phytochemical of DT seed revealed the presence of flavonoids, saponins, resins, alkaloids, and so forth. Studies in the recent past (Oyedemi et al., 2012; Unnikrishnan et al., 2013) reported that dietary flavonoids, alkaloids, saponins, tannins, and glycosides have antidiabetic potentials. These bioactive phytochemicals,

as reported in previous studies (Ivorra et al., 1989; Kameswara et al., 2003) may either singly or in synergy with one another be responsible for the significant glucose-lowering activity reported in the current study. The hypoglycemic effect of *D. tripetala* extract found in this study agrees with previous reports (Isegholi, 2015; Anioke et al., 2017).

In Table 3, the diabetic negative control rats showed a significantly (P≤0.01) elevated levels of MDA compared with normal control rats. The diabetic positive control group that received glibenclamide showed a significant (P≤0.05) decrease in MDA levels compared to diabetic control group. This could be due to an effective glycemic control of the drug thereby decreasing hyperglycemic induced lipid peroxidation; this finding is in support of the work done by Ahmed et al. (2006). The extract treated diabetic groups showed a significantly (P≤0.01) decreased lipid peroxidation as compared to the normal and diabetic control groups, 100 mg/kg of the extract had the lowest value for MDA as opposed to the 200 mg/kg of *D. tripetala* extract which had a more increased MDA level. This shows that the ability of the extract to attenuate lipid peroxidation is not dose dependent. This decrease can be due to the high antioxidant capacity of the extract (Moussa, 2008; Anioke et al., 2017). The increased levels of lipid peroxidation in group 5 can be associated with the presence of toxic pyrrolizidine alkaloids (Ahmed et al., 2006; Anaga et al., 2006). Hypoinsulinaemia in diabetes increases the activity of the enzyme fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids,

resulting in lipid peroxidation (Horie et al., 1981). The products of lipid peroxidation are harmful to most cells in the body and are associated with a variety of diseases, such as atherosclerosis and brain damage. The changes in the GSH-Px levels were not significant ($P > 0.05$), although the glibenclamide treated group had a decreased GSH-Px levels as compared to the diabetic negative and normal control group, this is in support of the work done by Lu et al. (2011). The diabetic negative control group had a lower GSH-Px level as compared to normal control. The extract groups had increased level of GSH-Px as compared to diabetic controls and this increase was dose dependent with the values of 200 mg/kg higher than 100 mg/kg. In diabetic patients, the auto-oxidation of glucose results in the formation of hydrogen peroxide which inactivates SOD and this accumulated hydrogen peroxide may be one of the explanations for decreased activity of SOD in type 2 diabetic patients (Nobar et al., 1999). The SOD activities were significantly increased in the glibenclamide group as compared to the diabetic negative control group. This could be due to effect of the drug on the attenuation of oxidative stress (Shinde et al., 2011). The SOD activities of the extract groups were increased in a dose dependent manner as compared to the diabetic negative control, which further shows the presence of phytochemical and antioxidant capacity of *D. tripetala*. The diabetic control groups had a mildly increased catalase activity as compared to the normal control. There was a significant increase in the extract treatment groups (200 mg/kg at $P < 0.05$). The CAT activities of the extract groups were increased in a dose dependent manner as compared to the normal control which further shows the presence of phytochemical and antioxidant capacity of *D. tripetala*. This finding is in support of the previous works done by Acworth et al. (1997). In untreated diabetic rats, percentage increase in body weight decreased significantly compared to the normal control, which may be attributed to increased degradation of structural protein due to damage to the intracellular signaling pathways implicated in maintaining the balance between protein synthesis and degradation (Newsholme et al., 2011; Hulmi et al., 2012). Interestingly, there was a significant increase in the body weight when compared with the untreated (negative control) group after treatment. This suggests that *D. tripetala* may have a bioactive potency like IGF-1, which increases protein synthesis in diabetes to restore muscle wasting through the activation of Akt/mTOR (protein kinase B/mammalian target of rapamycin) pathways (Glass, 2005; Zhang et al., 2014).

Conclusion

The present experimental study demonstrated anti-hyperglycemic potency of *D. tripetala* (pepper fruit) which is comparable with glibenclamide in the treatment of

diabetes. The potential antidiabetic properties linked with *D. tripetala* need to be therapeutically maximized to ameliorate the burden of diabetes and its complications in the society. Furthermore, the study shows that *D. tripetala* should be taken at an optimal dose of 100 mg/kg to prevent an increase in lipid peroxidation as seen at 200 mg/kg dose. There is a need for further investigation to elucidate the precise mechanism leading to the hypoglycemic effect present in *D. tripetala* fruits with the antidiabetic property observed in the study; also the lipid peroxidation present at higher doses should be investigated. Furthermore, glibenclamide which is the standard diabetic drug used in this study should be researched further to validate its glutathione peroxidase lowering effect and weight gain, and if consistent with this study, there is need for a review.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effectiveness of solid carriers on multiplication of bradyrhizobium mutant strain and nodulation of cowpea (*Vigna unguiculata* L)

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Effective nodulation of legume is usually solid carriers dependent. Peat, the most effective solid carrier is not readily available in Nigeria, hence, alternative solid carriers were evaluated with Bradyrhizobium strains using cowpea as a test crop. Mutant strains (USDA 3384 and USDA 3451) were evaluated in the laboratory using solid carriers and pot experiments. The survival (multiplication), infectivity and nodulating ability of the Bradyrhizobium mutant strains using cowpea as a test crop were assayed in peat, cowdung and composted maize cob. Mutant strains inoculated in the three solid carriers got to their peak of growth at 16th day of incubation. Peat significantly ($p < 0.05$) increased the population of both USDA 3384 and USDA 3451 compared to cowdung and composted maize cob. USDA 3451 inoculated in cowdung significantly ($p < 0.05$) increased multiplication of *Bradyrhizobium* mutant strain and nodulation of cowpea. Investigation shows it can be used as peat substitute when preparing inoculant. USDA 3384 inoculated in both composted maize cob and cowdung, respectively had consistent and significant positive effect on nodulation of cowpea and nodule dry weight. These results reveal their ability to substitute peat. Hence, blind use of alternative solid carriers for inoculant preparations may hinder high infectivity and optimal nodulation which could facilitate positive effects of nitrogen fixation in low N tropical soil.

Key words: Solid carriers, Bradyrhizobium, survival, infectivity, nodulation.

INTRODUCTION

Tropical soils are inherently low in fertility. Constant exposure of soil of this region to forces of degradation which causes rapid deterioration in their physical, chemical and biological properties is due to the

intensification of agricultural production (Obi and Ebo, 1995). Organic matter content and nutrient status especially related to nitrogen, which is largely important in crop production, are generally low in tropical soil (Lal

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and Kang, 1982; Henao and Baanante, 2006). The growing world population is presenting a challenge of adequate and balanced nutrition, thus, the need for sustainable crop production that will preserve the environment. The nitrogen reserve of agricultural soils must be replenished regularly in order to maintain crop production. Replacement of soil nitrogen can be accomplished by the addition of inorganic fertilizers and by biological nitrogen fixation (Giller et al., 2009).

More than 50% of inorganic N – fertilizer applied to remedy the problem of low soil nitrogen in the tropics polluted the environment (Ladha et al., 1998) through acidification of soil (Kennedy and Tchan, 1992) and nitrate pollution of ground water through leaching (Shrestha and Ladha, 1998). Under intensive agriculture, use of inorganic fertilizers alone has not been helpful because it aggravates soil degradation (Sharma and Mittra, 1991) due to loss of organic matter and resulting in soil acidity, nutrient imbalance and low crop yields (Ayoola and Makinde, 2007).

Biological nitrogen fixation (BNF) can be a major source of nitrogen to tropical soils especially when symbiotic N₂ – fixing systems are used. The contribution to soil nitrogen economy through this system is as high as 360 kg/ha (Bohlool et al., 1992). Biofertilizers, which are preparations containing living cells of efficient strain of microorganisms, such as *Rhizobium*, help crop plants to uptake nutrients (Jeyabal and Kuppswamy, 2001).

Problems associated with N₂-fixation in *rhizobium* - legumes symbiosis include inappropriate, specificity or insufficiency of strains of bradyrhizobium which can be corrected by inoculation of appropriate strains (Lindemann and Glover, 2003). Therefore the need to inoculate legumes with respect to specificity and sufficiency for effectiveness and infectivity have been observed and researchers on use of *Rhizobium* inoculants for production of grain legumes also reported its effectiveness in agronomic practice for ensuring adequate N nutrition of legumes, compared with the application of N fertilizer (Chianu et al., 2008).

Roy et al. (2010) discovered that population of *Bradyrhizobium* strains declined progressively shortly after introduction to the soil. This may be as a result of heterogenous and unpredictable environment of soil. For effective survival and multiplication of *rhizobium* strains, carriers are needed for inoculant preparation. This also helps to transfer sufficient number of desired *rhizobia* strain to target legumes (Brockwell and Bottomley, 1995). High water capacity, ability to foster multiplication in *rhizobium*, non-toxicity, easy to sterilize by autoclave or gamma irradiation, readily available and not expensive, pH buffering capacity and high adhesiveness for effective application on to seeds are characteristics of a good carrier, observed by Keyser et al. (1992). Research has discovered that peat has these qualities but has been on use for long, hence needs a substitute due to its unavailability in most countries.

Freire and Verneti (1999), confirmed that liquid carrier inoculants are good for mechanical sowing. Some locally available agricultural materials like filtermud, bagasse, sawdust, coffee husks, coir dust, charcoal dust and forest soil were investigated in Kenya to select those which had similar characteristics to peat (Kibunja, 1991). Filtermud was observed to increase the survival rate of *Rhizobium phaseoli* as determined by plate count method.

Rhizobium strains differ in their ability to utilize whatever carbon-compound and other nutrient solid carriers, due to their different rate of metabolizing these carriers. A particular *Bradyrhizobium* strain can perform well in a particular solid carrier but not in another over a period of time. Apparently, there should be strict investigation on the preference of a particular *rhizobium* strain for different alternative solid carriers as substitute for peat. This research covered both laboratory and pot investigations of *Bradyrhizobia* strains survival, multiplication and their infectivity and nodulating ability in solid carriers using cowpea as a test crop.

MATERIALS AND METHODS

Soil sample

Low – N soil, used for the pot experiment (so as to relate some of the variables assessed) were taken at a depth of 0 -15 cm and were obtained from the Teaching and Research Farm, Department of Agronomy and Landscape Design, Babcock University, Ilishan Remo, Ogun State. Low – N soil's physical, chemical and biological characteristics were as follows: 720 g/kg Sand, 114 g/kg silt, 166 g/kg clay; pH in (H₂O) 5.7; 14 g/kg organic carbon; 8 mg/kg extractable P (Bray 1) soils; 1.0 g/kg total N and Exchangeable Ca, Mg, K were 14.6, 1.1, 3.0 Cmol/kg respectively while the indigenous *rhizobium* population was 10 viable cells/g soil (1×10¹ cfu/g).

Experimental design

The experiment was a completely randomised design replicated four times with a 3 × 2 factorial combination comprising of two *bradyrhizobium* strains (USDA 3384 and USDA 3451) and three solid carrier (peat, composted maize cob and cowdung).

The solid rhizobium carriers

The solid *Rhizobium* carrier namely peat (imported conventional carrier) was obtained from International Institute of Tropical Agriculture (IITA). Other two locally sourced possible alternative carriers, cowdung and composted maize cob were obtained from the Teaching and Research Farm of the Department of Agronomy and Landscape Design, Babcock University. They were autoclaved at 121°C for 30 min and were analysed for water holding capacity and other chemical properties as shown in Table 1

Determination of growth of bradyrhizobium mutant strains in solid carriers

Two parent *Bradyrhizobium* strains (obtained from Soil Microbiology Laboratory, IITA) which are the most infective (ability to nodulate) and effective (ability to fix nitrogen) in nodulation were developed

Table 1. Water holding capacity and other chemical properties of the three solid carriers.

Properties	Peat	Composted maize cob	Cowdung
Water holding capacity (%)	53	75	75
pH in H ₂ O	6.20	6.72	9.24
Total nitrogen (%)	2.35	4.51	5.03
Organic carbon (%)	61.78	37.52	23.78
Total P (%)	0.28	0.95	0.31
Ca (%)	0.01	3.59	0.71
Mg (%)	0.31	1.56	1.11
K (%)	0.62	0.52	3.13
Na (mg/g)	555.88	967.30	762.96

into mutant strains by screening them using Mannitol Yeast Extract Agar (A selective medium for isolation and quantification of bradyrhizobium spp) with various antibiotics concentrations (Tas et al., 1996). Three ratio two (3:2) w/v of each of the six treatments, which was the mixture of 240 g solid *bradyrhizobium* carrier and 160 ml of seven day old broth culture of the two mutant strains, were introduced into sterilized flasks and incubated at a temperature of $24 \pm 2^\circ\text{C}$ in a lab laminar flow. At 8, 16 and 24 days after incubation, 1.0 g of the mixtures was aseptically taken to make serial dilution (10^{-2} to 10^{-8}) according to the procedure of Vincent (1970). For the determination of the mutant (isolate) population in the carriers, 1.0 ml of each of 10^{-7} and 10^{-8} dilution were pipetted out in triplicates into sterile Petri dishes and 15 ml of melted MYEA was added (Packialakshmi and Riswana, 2014). The diluent and the agar were thoroughly mixed before the plates were incubated invertedly (two to five days incubation) at 28°C .

Effect of solid carriers/mutant strain mixture on nodulation of cowpea

Treatment combination was formulated from the solid carriers and mutant factor and they were assessed in terms of root nodulation (number and dry weight), and %strain nodule occupancy of *Vigna unguiculata* species.

One hundred and sixty millilitres (160 ml) of a seven days old broth culture of the infective mutant (isolate) for the two different stains USDA 3384 (3.0×10^{10} cell/ml) and USDA 3451 (3.3×10^{10} cell/ml) were aseptically dispensed into 240 g of each of the three sterilized solid carriers (2:1 w/v), respectively. These mixtures were incubated at room temperature for 16 days before use. Basal rate of urea (20 kg N/ha), muriate of potash (30 kg K/ha) and single super phosphate (40 kg P/ha) were thoroughly mixed with bulk (640 kg) air dried soil passed through 2 mm sieve. Five kilogrammes (5 kg) portion of the soil was introduced into pots perforated basally to facilitate drainage of any excess water.

Surface sterilized cowpea seeds of the Ife Bimpe variety were used for this experiment which was moistened with 30% gum arabic. The seeds were coated with each of the three solid carrier / mutant treatment and were spread aseptically inside the laminar flow to dry overnight. After this time, four seeds were sown 1 to 1.5 cm deep into the soil in each pot. The pots were watered when necessary after seed sowing. Ten days after sowing, the seedlings were thinned to two per pot. To ensure high *bradyrhizobial* population in the rhizosphere, 4 ml of *rhizobial* broth culture of each mutant was dispensed per seedling in each pot.

Measurement and harvest

Number of plant nodules were assessed two weeks after sowing

(WAS) while the plant nodule dry weight and root dry matter yield were assessed at 6 and 8 WAS. Eight nodules were chosen randomly and detached from each of the treatment at 8 WAS, they were surface sterilized and used for typing back nodule strain. Each of the nodules chosen was crushed in 1 ml of sterile saline water (0.85%) and a loopful of the suspension was cultured using the spread plate method on the antibiotic amended agar. *Bradyrhizobial* growth was examined after incubation for five days at 28°C . The number of nodules that formed colonies per treatment in the plates were counted and data collected was used for the computation of percent strain nodule occupancy. This was equated to the proportion of nodules formed by the mutant strain on the cowpea species used (Typing back).

Statistical analyses

Data collected were subjected to analysis of variance (ANOVA) and descriptive statistics while treatment means were separated using Duncan multiple range test (DMRT) at 5% probability level.

RESULTS AND DISCUSSION

Water holding capacity of peat was the lowest of the three carriers. Composted maize cob and cowdung were similar in their water holding capacity (Table 1). pH showed that cowdung was alkaline, composted maize cob was near neutral while peat was slightly acidic. Total nitrogen was lowest in peat and highest in cowdung while composted maize cob was in between but almost twice that of peat. Organic carbon was highest in peat, lowest in cowdung and intermediate in composted maize cob. Total P, Mg and Na were highest in composted maize cob, lowest in peat and intermediate in cowdung. Highest Ca was obtained in composted maize cob followed by cowdung with the observed in lowest in peat. K was highest in cowdung followed by peat, the lowest was observed in composted maize cob.

At 8 and 16 days of post - inoculation, the trend of population of the two isolates were similar in all the three solid carriers, but at 24 days post - inoculation, composted maize cob enhanced the population of USDA 3541 more than cowdung, whereas, the trend of peat > cowdung > composted maize cob was observed with USDA 3384. In both strains, the highest population of

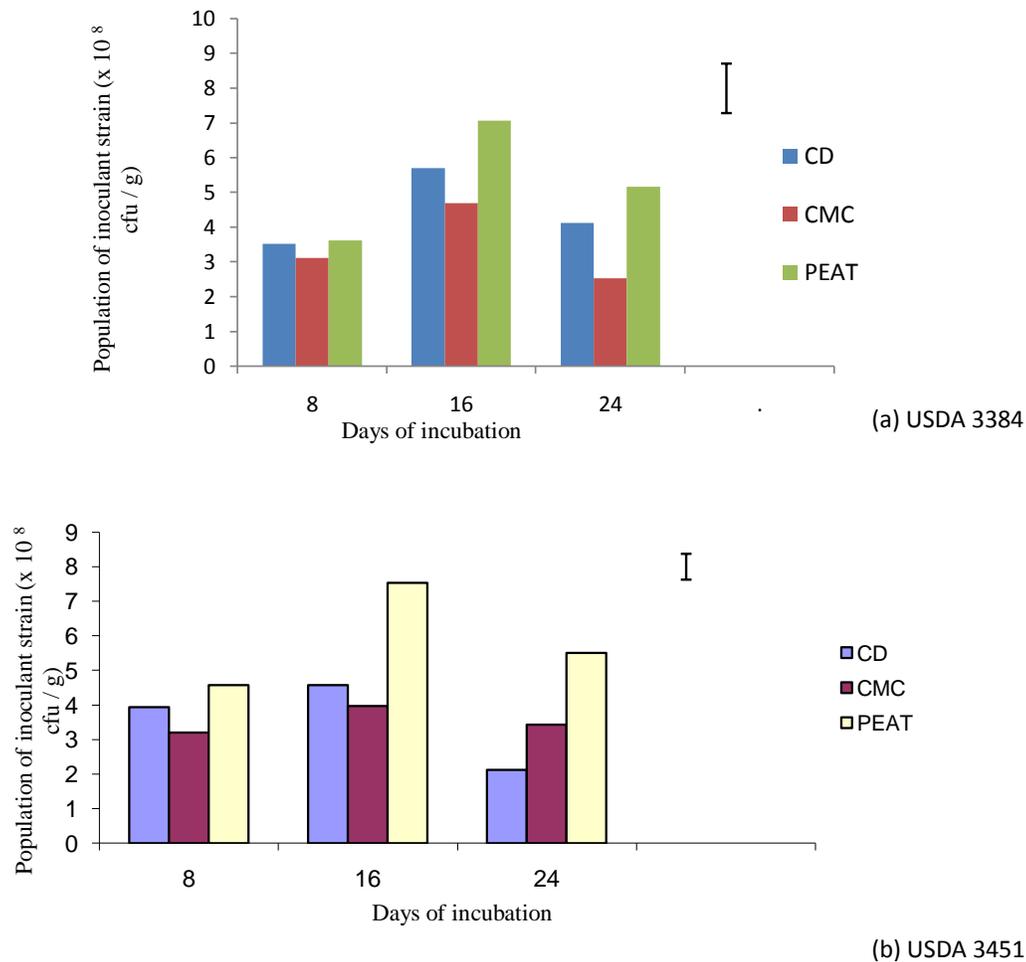


Figure 1. Effect of incubation period of *Bradyrhizobium* strain in peat, composted maize cob and cowdung solid carriers. LSD (0.05). CD = cowdung; CMC = composted maize cob; USDA = United State Department of Agriculture; Bar represent Least Significant Different (LSD).

Bradyrhizobium was obtained at 16th day after incubation (Figure 1). Hence, the strains differ in their ability to grow in any given medium which is facilitated by their environmental and nutritional needs (Richardson, 2001). Figueiredo et al. (1992) and Khavazi et al. (2007) also reported that the chemical and physical characteristics of alternative carrier materials determines the maintenance of initial concentrations of *Bradyrhizobium* cells in inoculants. It is evident that the ability of the mutant strains to grow in a medium depending on their specific need significantly resulted in their differential growth. None of the solid carriers used was significantly higher compared to peat in terms of promoting mutant survival and growth in carriers. This is in line with the findings of Brockwell and Bottomley (1995), who observed that peat was the most suitable solid carrier for inoculant production.

At two weeks after sowing, the highest nodule number was obtained in cowpea when composted maize cob was

used as solid carrier for strain USDA 3384 while the lowest was also obtained using composted maize cob as solid carrier for strain USDA 3451 (Table 2). Number of nodules obtained with peat and cowdung were not significantly different. At 4 weeks after sowing, the highest nodule number was obtained when cowdung was used as carrier with mutant strain USDA 3384 which was not significantly different from other treatments except composted maize cob and cowdung with strain USDA 3451. At six weeks after sowing, nodule numbers reduced compared to those obtained at 4 weeks after sowing. Despite the highest number of nodules obtained with cowdung using strain USDA 3451, there was no significant difference among the treatments. At eight weeks after sowing, the highest number of nodules was obtained on cowdung with strain USDA 3384, but this was not significantly different from composted maize cob with the same strain as well as cowdung with strain USDA 3451. Peat as solid carrier at the early stage of cowpea

Table 2. Effect of Bradyrhizobium mutant strains with three solid carriers on number of nodules/plant of cowpea at different weeks after sowing.

Treatment	Weeks afer sowing			
	2	4	6	8
C1M1	54.3 ^b	92.8 ^a	71.5 ^{ab}	76.8 ^b
C2M1	64.3 ^a	94.1 ^a	72.6 ^{ab}	92.6 ^{ab}
C3M1	52.8 ^b	96.0 ^a	65.1 ^{abc}	106.3 ^a
C1M2	54.8 ^b	94.0 ^a	58.8 ^{abc}	72.4 ^b
C2M2	22.9 ^c	50.9 ^{bc}	58.6 ^{abc}	40.6 ^c
C3M2	52.3 ^b	60.0 ^b	76.0 ^a	97.0 ^{ab}

Means with same letter(s) in a column are not significantly different at 5% level of probability according to Duncan multiple range test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize cob/mutant strain 1; C3M1 = cowdung/mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/mutant strain 2; C3M2 = cowdung/ mutant strain 2; Mutant strain 2 = USDA 3451.

Table 3. Effect of Bradyrhizobium mutant strains with three solid carriers on dry weight of nodules (g) of cowpea at different weeks after sowing.

Treatment	Weeks after sowing		
	4	6	8
C1M1	2.2 ^a	1.1 ^a	2.0 ^{ab}
C2M1	2.1 ^{ab}	1.2 ^a	2.1 ^a
C3M1	1.6 ^c	1.1 ^a	1.8 ^{ab}
C1M2	1.6 ^{bc}	1.3 ^a	2.1 ^a
C2M2	1.0 ^d	1.1 ^a	1.2 ^{bc}
C2M3	1.6 ^c	1.1 ^a	1.8 ^{ab}

Means with same letter (s) in a column are not significantly different at 5% level of probability according to Duncan multiple range test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize; cob/mutant strain 1; C3M1 = cowdung/mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/mutant strain 2; C3M2 = cowdung/mutant strain 2; mutant strain 2 = USDA 3451.

growth did not provide a significantly higher number of nodules compared to other treatments. The increase in performance observed in cowdung was similar to the findings of Dharma (1996) who reported that farmyard manure (cowdung) has the ability to stimulate the activities of microorganisms which can help make nutrients readily available to plant, thus increasing cowpea nodulation. This result also corresponds with the earlier work where it was discovered that rhizobium inoculation can improve nodulation, N₂ fixation and yield (Delić et al., 2009)

At six weeks after sowing, all treatments were significantly different with respect to nodule dry weight (Table 3). At four weeks after sowing, the highest nodule dry weight was obtained under the peat as carrier with mutant strain USDA 3384, while the lowest was observed on composted maize cob with mutant strain USDA 3451. The lowest nodule dry weight was significantly lower compared to other nodule dry weights, while the highest nodule dry weight was also significantly higher compared to other treatments except composted maize cob with

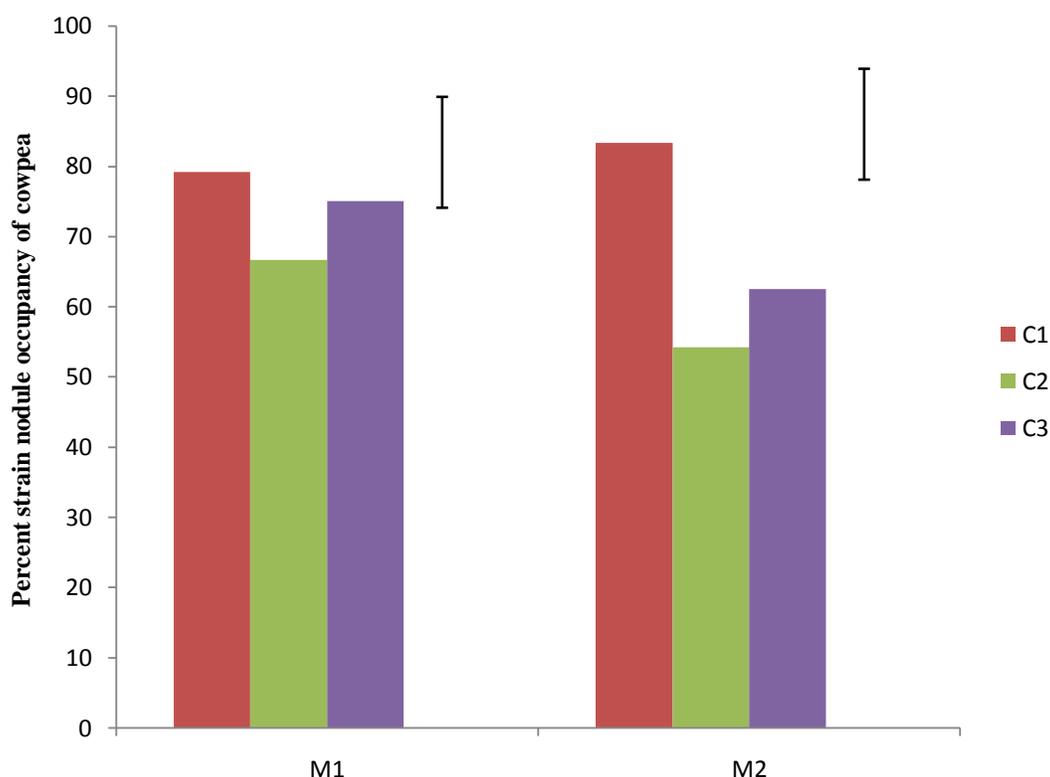
mutant strain USDA 3384. At eight weeks after sowing, only composted maize cob with mutant strain USDA 3451 was significantly lower compared to treatments with the highest nodule dry weight.

The root dry weight of cowpea under peat as solid carrier with mutant strain USDA 3451 was the highest at six weeks after sowing but it was only significantly higher than the root dry weight of plants under composted maize cob with mutant strain 3451. Other treatments were not significantly different (Table 4). At eight weeks after sowing, the composted maize cob with USDA 3384 inoculated plant had the highest root dry weight which was significantly higher than other treatments except peat with USDA 3451. Other treatments were not significantly different. Increase in root dry weight in composted maize cob and cowdung could be due to their high nitrogen content (Table 1). Similar work was reported by Shaukat (1994) on nitrogen effect on root weight. Growth and development of crops depend largely on development of root system hence the need for major macro and micro nutrients which solid carriers can provide is high

Table 4. Effect of Bradyrhizobium mutant strains with three solid carriers on root dry weight/plant of cowpea at different weeks after sowing.

Treatments	Root dry weight /plant	
	6 Weeks after sowing	8 Weeks after sowing
C1M1	1.9 ^a	2.1 ^{bc}
C2M1	1.6 ^{ab}	3.0 ^a
C3M1	1.8 ^a	2.0 ^{bc}
C1M2	2.0 ^a	2.8 ^{ab}
C2M2	1.2 ^b	1.8 ^{bc}
C2M	1.7 ^{ab}	2.1 ^{bc}

Means with same letter(s) in a column are not significantly different at 5% level of probability according to Duncan Multiple Range Test (DMRT). C1M1 = peat/mutant strain 1; C2M1 = composted maize cob/ mutant strain 1; C3M1 = cowdung/ mutant strain 1; mutant strain 1 = USDA 3384; C1M2 = peat/mutant strain 2; C2M2 = composted maize cob/ mutant strain 2; C3M2 = cowdung/ mutant strain 2; mutant strain 2 = USDA 3451.

**Figure 2.** Effect of Bradyrhizobium mutant strains with three solid carriers on nodule occupancy of cowpea. LSD (0.05); C1 = peat; C2 = composted maize cob; C3 = cowdung; Bar represent Least Significant Difference; M1 = Mutant strain USDA3384; M2= Mutant strain USDA3451.

(Veeramani and Subrahmaniyan, 2011).

The relative competitiveness or contribution of the introduced strain to legume nodulation in the presence of soil native *Rhizobium* can be marked by an index known as percent strain nodule occupancy. USDA 3384 inoculated with peat was responsible for 79.17% strain nodule occupancy compared to USDA 3384 inoculated

with cowdung and composted maize cob, which resulted to 75.0 and 66.67% strain nodule occupancy, respectively (Figure 2). This might be due to the fact that rhizobium is required in sufficient number for optimal nodulation and efficient nitrogen fixation, especially when a legume is being cultivated on a particular soil or land for the first time (Catroux et al., 2001). Strain nodule occupancy

significantly increased up to 83.33% when USDA 3451 was inoculated with peat compared to USDA 3451 inoculated with cowdung, which was responsible for 62.50% strain nodule occupancy and USDA 3451 inoculated with composted maize cob, which resulted in 54.17% strain nodule occupancy. Peat maintained its superiority as the most suitable carrier (Smith, 1992), resulting in best performance in respect to its percentage strain nodule occupancy. However, cowdung also showed that it can be a suitable peat replacement when USDA 3451 is used, compared to composted maize cob (Stephens and Rask, 2000).

Most of the percent strain nodule occupancy obtained in this experiment fall within 47 to 80% as reported by Jensen and Sorensen (1987). The high percent strain nodule occupancy obtained indicates that the mutant strains (USDA 3384 and USDA 3451) used for inoculant preparation in this experiment possess the ability to compete favourably with indigenous rhizobium present in the soil and are capable of forming nodules on a plant host (Martensson, 1989), hence have high ability of fixing nitrogen with their target host legume (Howieson et al., 2000). An obvious explanation for this could be because the introduced *Bradyrhizobium* strains used for this experiment which was higher in population (compared to native rhizobium which was as low as 1×10^1 cfu/g in population) most probably enhanced by use of solid carriers resulted to high percent strain nodule occupancy, since use of carrier based inoculant determines the transfer of the desired *rhizobium* strain with sufficient numbers to targeted legume (Brockwell and Bottomley, 1995).

Conclusion

The use of the three solid carriers for *Bradyrhizobium* inoculation showed a promising outcome with respect to infectivity and nodulation, dry nodule weight, root dry weight and percent strain nodule occupancy in cowpea. Both composted maize cob and cowdung showed qualities as peat (suitable carrier according to Brockwell and Bottomley (1995) substitutes when USDA 3384 strain was used for inoculation with respect to all the parameters, while cow dung was preferable as a peat substitute when USDA 3451 was used for inoculant preparation with respect to the majority of estimated parameters. *Bradyrhizobium* strains' compatibility with solid carrier should be ascertained before using such solid carrier for inoculant preparation. This will help to avoid blind use of solid carrier which can cause inefficiency of introduced strains when used for inoculation of legumes.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chemical and physico-chemical characterization of seeds of melon cultivars: Valenciano and Pele de Sapo, cultivated in the Amazon

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The aim of this work was to perform chemical and physical-chemical analyzes of the oil of the cultivars Valenciano Amarelo melon (VA) and Pele de Sapo melon (PS) (*Cucumis melo* L.) produced in Boa Vista, Roraima, Brazil, by GC -FID, IR, ¹H NMR and the thermogravimetric behavior TG/DTG as well as yield of that obtained oil. Thus, the oil yield of Valenciano melon seeds was 28.47% and that of Pele de Sapo melon was 26.41%. The chemical composition of the oils of the two cultivars presented major fatty acids, such as palmitic acid, linoleic acid (57.5-59.4%) and oleic acid (22.8-23.3%). The physico-chemical characterization of these oils showed for Valenciano melon and Pele de Sapo melon, respectively, 125.02 and 120.96 g I₂ 100 g⁻¹ for the iodine value and 187.80 and 185.65 mg KOH g⁻¹ for saponification index. The behavior of the thermal degradation by TG/DTG of the melon seeds oils VA and PS occurred between 341.08 and 522.03°C, and 334.83 and 524.29°C, respectively.

Key words: Oleic acid, cucurbitaceas, biotechnology, essential fatty acids.

INTRODUCTION

Melon is one of the olerícolas belonging to the family Cucurbitaceae, genus *Cucumis*. It is an herbaceous and

creeping plant of African origin (Bisognin, 2002), cultivated mainly in India and in tropical countries, with

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varieties and cultivars (Mansouri et al., 2015), introduced in Brazil by the Europeans in the decade of the 60s (Frizzone et al., 2005).

Melon cultivation is carried out in regions with a very dry and bright climate good drainage soil and temperature ranging from 25 to 35 °C. The temperature and luminosity favor the sweetest and tasty fruits, as it helps the sugar production. The variations of melon grown in Brazil, have pleasant organoleptic characteristics, highlighting the pleasant aroma (Senar, 2007).

Melon cultivars found in Brazil are Honey Dew, Pele-de-sapo, Orange Flesh, Cantaloupe, Gália, Orange and an AF-522 hybrid commercially classified as a yellow melon type (Melo et al., 2000). The main melon growing regions for the year 2013 were the San Francisco Valley (Pernambuco and Bahia), Rio Grande do Norte and Ceará in a total of land area of 14,950 hectares (2,950 in the São Francisco Valley and 12,000 in the Rio Grande do Norte and Ceará) from August /2013 to March / 2014 (Nascimento, 2014). Among the melons cultivated in Brazil, the cultivars of the odorless group, represented by the Valenciano and Pele-de-sapo types, are preferred by the producers, totaling about 90% of the planted area (Nunes et al., 2005).

In the food industry, after the use of fruit pulps the seeds of these fruits are discarded in the environment. This is due to the lack of alternatives to the use of these vegetable residues, being used as organic fertilizers or animal feed, without adequate treatment (Miguel et al., 2008). These seeds could be used as an alternative source of socioeconomic value and by generating employment and income through vegetable (Kobori and Jorge, 2005; Uchoa et al., 2008).

In this work, the chemical composition and physicochemical properties of fatty acids extracted from the seeds of two varieties of melon (Valenciano and Pele-de-sapo) cultivated in the State of Roraima in Brazil were studied in order to be used as a biotechnological source for the production of other derivative products.

MATERIALS AND METHODS

Material and extraction of oil

Samples of the two melon cultivars were collected at the producer's fair in Boa Vista-Roraima (Brazil) in March 2015, in total 10 melons of each species with good appearance and in a ripe state suitable for consumption and taken to the Laboratory of Environmental Chemistry of the Graduate Program in Chemistry of the Federal University of Roraima the seeds were separated, washed with distilled water and dried in an air circulating oven at 50 °C for 48 hours until constant weight. After drying the seeds were ground and sieved in a 30-40 mesh sieve. The extraction of oil, was done in a Soxhlet extractor using cellulose cartridges covered with hydrophilic cotton and anhydrous sodium sulfate to control excessive humidity (Jorge and Luzia, 2012).

The solvent was hexane, the contact time between the solvent and the sample was 3 hours. The solvent was removed using a

rotary evaporator to give melon seeds oil as the final product. The reagents and solvents were in analytical purity in all procedures performed. The oils were stored in amber flasks in an atmosphere of gaseous nitrogen in order to protect the oil and conserve its properties. These were refrigerated until further analysis.

Profile of fatty acids by CG-FID

Free fatty acids were measured by Gas Chromatography using HP7820A (Agilent) system equipped with flame ionization detector. An Innowax column (HP) 15 m × 0.25 mm × 0.20 µm was used and the following temperature gradient: 100 °C min and 0.7 °C min⁻¹ up to 240 °C; injector (1/30 split) to 250 and 260 °C detector. Hydrogen was used as carrier gas (3 mL min⁻¹) and injection volume was 1 µL. The data acquisition program used was EZChrom Elite Compact (Agilent). The peaks were identified using FAME Mix C14-C22, CRM18917 Supelco fatty acid methyl esters standard (Christie, 1989).

Physicochemical properties of the oil by ¹H-RMN

In order to determine the physico-chemical properties of melon oil, the values of ¹H-RMN spectra, previously studied by Carneiro (2005) and Reda and Carneiro (2006) were utilized.

Thermal analyses (TG/DTG) were conducted on Shimadzu 60 DTG equipment using approximately 10 mg heated sample in an alumina crucible. The tests were performed with a heating rate of 10°C min⁻¹, from room temperature to 900°C, under nitrogen gas atmosphere (flow 50 mL.min⁻¹).

RESULTS AND DISCUSSION

Extraction yield

Table 1 shows the yields of the two varieties of melon studied in this work compared to the literature. The data are expressed as the mean value of the three readings with the standard deviation.

The density of the Valenciano melon seed oils was about 0.90 and 0.86 mg ml⁻¹, respectively. The values found in the seeds oil of the melon cultivars studied was in the range proposed by the literature, as shown by the yields of the oil of the Yellow Melon (Malacrida et al., 2007) and extraction of the Japanese Melon (Melo et al., 2007).

Among the cultivars studied, the Valenciano melon seeds had higher yields compared to the *Pele de Sapo* melon species. The oils obtained were yellowish with a sweet odor.

Determination of fatty acids in seeds using CG-FID

In Table 2, the fatty acid profile for the seeds of the two melon varieties compared to the literature is presented. The oils of the seeds of the cultivars Valenciano Melon and *Pele de Sapo* Melon, respectively, show an average composition of 81.00 and 83.00% for unsaturated fatty acids and 19.00 and 17.00% for saturated fatty acids.

Table 1. Oil yields for *Valenciano* and *Pele Sapo* melons.

Sample	Valenciano melon	Pele de Sapo melon	Melão Amarelo (Malacrida et al., 2007)	Melão Japonês (Melo et al., 2007)
Yield (%)	28.47 ± 0.10	26.41 ± 0.09	25,2 ± 0,6	29

Table 2. Composition of fatty acids in the oils of the seeds of two melon cultivars by gas chromatography in comparison to the literature.

Fatty acid	Valenciano melon (%)	Pele de Sapo melon (%)	P.E. (%)	A (%)	ANVISA
Miristic acids (C _{14:0})	0.1	0.1	0.22	0.13	< 0.3
Palmitic acid (C _{16:0})	10.2	9.3	11.08	28.29	5.5 - 11.0
Margaric acid (C _{17:0})	0.1	0.1	-	-	-
Oleic acids (C _{18:1})	22.8	23.3	19.32	49.74	12.0 - 28.0
Linoleic acids (C _{18:2})	57.5	59.4	40.19	7.57	58.0 - 78.0
Linolenic acids (C _{18:3})	0.7	0.3	0.61	1.49	< 1.0
Araquidic acids (C _{20:0})	0.3	0.2	-	-	< 1.0
Behenic acids (C _{22:0})	0.3	-	-	-	-
Other	8.0	13.1	0.42	1.49	-
Saturated	19.00	17.00	15.67	39.09	-
Unsaturated	81.00	83.00	84.3	60.91	-

The values obtained by the CG-FID for the composition of saturated fatty acids of the seeds oils of the cultivars *Valenciano* Melon and *Pele de Sapo* Melon shown in Table 2, present a very close composition with *Passiflora edullis* (Silva, 2011) and quite different from *Andiroba* (Farias, 2013).

Among the unsaturated fatty acids, the oils of the seeds of the cultivars *Valenciano* Melon, *Pele de Sapo* Melon and *Passiflora edullis* (Silva, 2011), the ω -9 acid presented very close values, whereas in *Andiroba* seed oil (Farias, 2013), the value found was higher than the others. The ω -6 oil of the seeds of the cultivars *Melão Valenciano* Amarelo and *Melão Pele de Sapo* presented values similar to those of the oil of the passion fruit *Passiflora edullis* (Silva, 2011), whereas in *Andiroba* seed oil (Farias, 2013), the value found was lower than the others.

A high content of linoleic acid was observed in melon seed oils which may characterize this oil as having nutritional properties and beneficial physiological effects in the prevention of diseases such as cancer and coronary heart disease (Azhari et al., 2014). The ω -3 oil of the seeds of the *Melão Valenciano* Amarelo and *Passiflora edullis* (Silva, 2011) seeds were similar. For the oil of the seeds of the cultivar *Melão Pele de Sapo*, the value obtained was lower than the former two, while for the *Andiroba* seed oil (Farias, 2013) the value found is superior to all.

As for the saturated fatty acid composition, palmitic acid presented the highest concentration followed by myristic acid for the oils studied and those in the literature.

Thermogravimetric analysis of melon seeds

Figures 1 and 2 present the TG / DTG curves of the melon seed oil where there is only one mass loss stage attributed to the volatilization and/or decomposition of triacylglycerides.

In the temperature range of 210 to 330°C, a low and continuous mass loss is observed. However, higher mass loss occurs at approximately 390°C, which can be attributed to the volatilization and/or decomposition of triacylglycerides (Garcia et al., 2004).

It was observed through the TG/DTG curves that the seed oils of the two melon species (Figure 1 and 2) undergo decomposition at temperatures below the stability temperatures of the oils. The oils analyzed presented an increasing order of stability as described below: *Melão Pele de Sapo* and *Melão Valenciano* Amarelo.

The analyzed oils had similar profiles with mass loss of 99.82 to 99.78%, where the process of volatilization and/or thermal decomposition occurred in a single stage, between 334 and 341°C, with a final temperature between 522 and 527°C (Table 3).

This similarity was observed in the curves profiles between the compositions of fatty acids in the oils of melon seeds which mainly present linoleic and oleic acids also known as omega-6 and omega-9 (Antolin and Meneses, 2000).

In comparison with other species such as *Andiroba* oil, a loss of mass of 96.16% was observed, while grape and soybean oils presented 97.02 and 94.49% of loss,

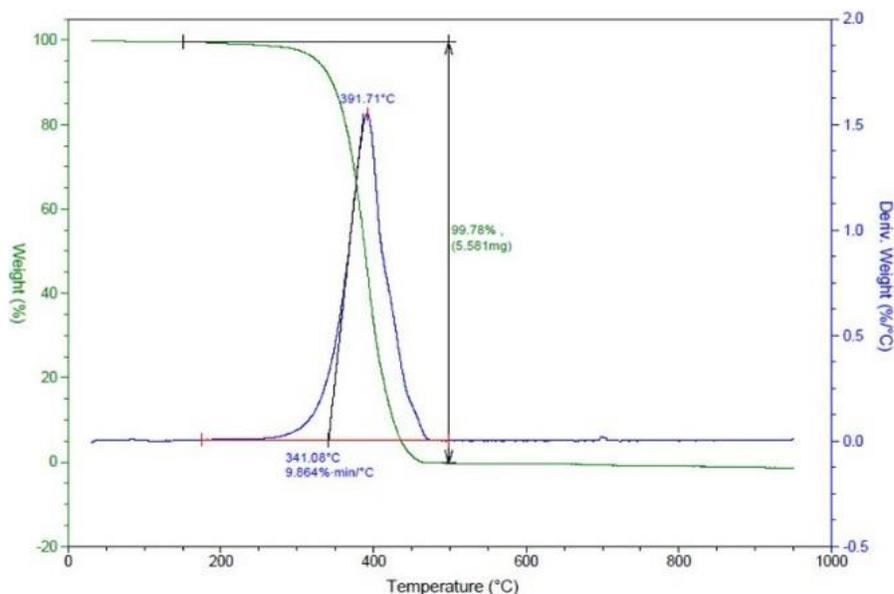


Figure 1. TG/DTG Yellow Valenciano Melon.

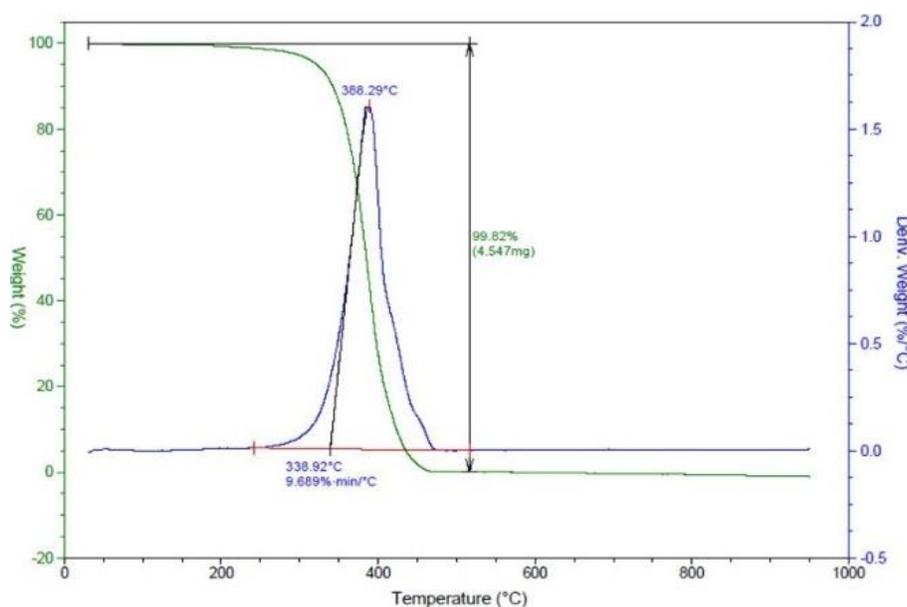


Figure 2. TG/DTG Pele-de-sapo Melon.

respectively, where the process of volatilization and/or thermal decomposition of the Andiroba oil was produced in the stages of 332.90°C with a final temperature of 641.78°C, and the Andiroba oil presented a thermal stability within the range found for the grape and soybean oils studied by Barreto (2009).

The profile of the curve of Figures 1 and 2 is due to the similarity between fatty acid compositions of the oils, which mainly present palmitic and oleic acid (Antolin and

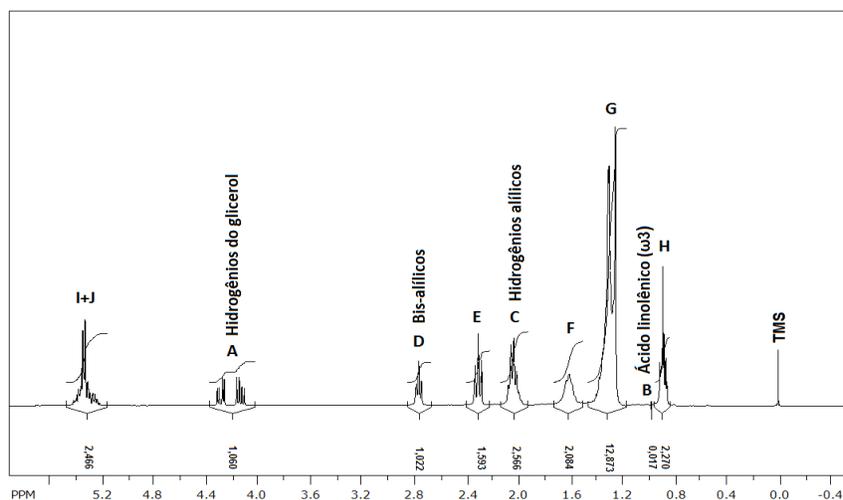
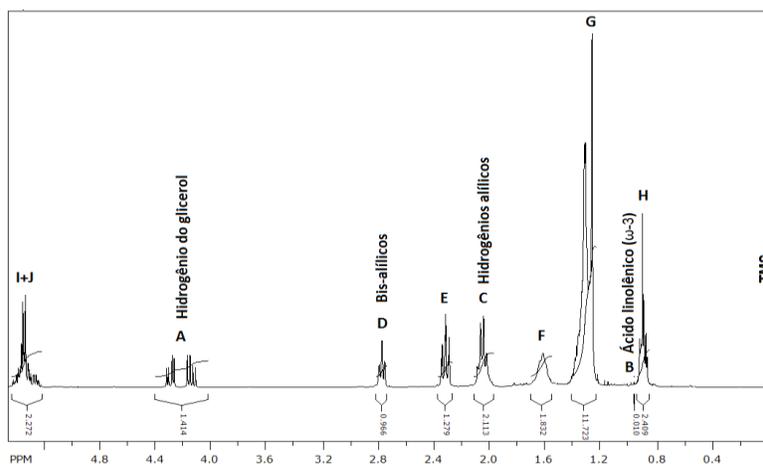
Meneses, 2000).

Samples of melon seed oil were found to follow a pattern, where the degradation started at 310°C and ended at approximately 527°C. At 527°C, mass loss of approximately 99.9% was found.

The results obtained by TG/DTG in nitrogen atmosphere showed that the thermal behavior of the oils of melon seeds was similar, especially with respect to the beginning and end temperatures of the degradation, for all the samples.

Table 3. Values for the different initial and final temperatures, ell as the values of mass loss obtained the TG/DTG curves.

Sample	T _i (°C)	T _f (°C)	Weight loss (%)
Valenciano melon	341.08	522.03	99.78
Peleda Sapo melon	334.83	524.29	99.82
Andiroba	332.90	522.93	96.16
White Niagara grape (Barreto, 2009)	345.01	522.93	97.02
Commercial soybean oil (Barreto, 2009)	324.49	527.50	94.49

**Figure 3.** ¹H-NMR spectrum of *Valenciano* melon seeds.**Figure 4.** ¹H-NMR spectrum of *Pele Sapo* melon seeds.

Physicochemical properties of melon seeds by ¹H-NMR

The values of ¹H Nuclear Magnetic Resonance spectra (Figures 3 and 4) were used to obtain the acid number, iodine, and saponification of the average molecular mass of the seeds of the three melon cultivars according to the

equations previously studied by Reda and Carneiro (2006).

The iodine index indicates the degree of fatty acid instabilities present in vegetable oils; the greater the degree of saturation the more the oil becomes unfit for human consumption (Reda and Carneiro, 2006). The oil of the seeds of the two melon cultivars presented an

Table 4. Physical-chemical characterization of the oil of the seeds of two melon cultivars, in relation to the literature.

Physical-chemical parameter	(mg I ₂ 100g ⁻¹) (II)	(mg KOH g ⁻¹) (IS)	(mg KOH g ⁻¹) (IA)	(g mol ⁻¹) (MM)	R _{o,A}
Valenciano Melon	125.02	187.78	0.73	893.33	3.85
Pele-de-sapo Melon	120.96	185.65	0.75	902.33	3.64
Crimson Sweet(Citrullus lanatus)	101.04	193.47	0.17	869.14	0.93
Andiroba(Caraca guianensis)	48.41	161.67	1.84	1004.02	0.27

iodine content of 125.02 mg I₂ 100 g⁻¹ for the oil of the *Valenciano* Melon seeds and 120.96 mg I₂ 100 g⁻¹ for the oil of the *Pele de Sapo* Melon seeds.

In Table 4, the values of the oils of the seeds of the two melon cultivars were higher than that the Crimson Sweet watermelon seeds (*Citrullus lanatus*) studied by Holanda, (2013) and the oil of *Caraca guianensis* studied by Farias (2013). However, due to the low values of saturation index expected, it leads to the iodine index values being within the established values.

The saponification index is an approximate measure to establish the average molecular weight, being indicative of the presence of high and low molecular fatty acids (Solomons and Fryhle 2012; Morreto and Fett, 2008). The oil of the seeds of the two melon cultivars presented a saponification index of 187.78 mg KOH g⁻¹ for the oil of the *Valenciano* melon seeds and 185.65 mg KOH g⁻¹ for the oil of the *Pele-de-sapo* Melon seeds. The value found for the saponification index indicates that the oil of the *Crimson Sweet* watermelon seeds has a low degree of deterioration (Holanda, 2013).

The values found for the saponification index of the oils of the seeds of the two melon cultivars were lower than the oil of the *Crimson Sweet* watermelon seeds studied by Holanda (2013) and higher than the *Caraca guianensis* oil studied by Farias (2013).

The oil of the seeds of the two melon cultivars had an acid value of 0.73 mg KOH g⁻¹ for the oil of the *Valenciano* Melon seeds and 0.75 mg KOH g⁻¹ for the oil of the *Pele-de-sapo* Melon seeds respectively. The observed values from this study were higher acidity index than the value found in *Crimson Sweet* watermelon oil (Holanda, 2013) and lower than the value found in *Caraca guianensis* oil by Farias (2013). However, the acid values of the melon oils remain between the maximum value established by ANVISA, (2000) which is 0.6%. Since the acidity index establishes the degree of conservation of an oil, the values found indicate that the oils were well conserved for further analysis.

A value greater than or equal to 0.66 indicates that vegetable oil is suitable for human consumption. This parameter needs to be calculated to obtain the acidity index of a vegetable oil (Reda et al., 2005). The oil of the seeds of the two melon cultivars presented oleophilic/aliphatic hydrogen ratio of 3.85 for the oil of the melon yellow seeds, 3.64 for the oil of the *Pele de Sapo* Melon

seeds.

Table 4 shows the values obtained for the oleophilic/aliphatic hydrogen R_o ratio of the oils of the seeds of the two melon cultivars, which were higher than the value found in *Crimson Sweet* watermelon oil (Holanda, 2013) and the value found in the oil of *Caraca guianensis* (Farias, 2013). The values indicate that the oils of the melon seeds are suitable for human consumption.

The average molecular mass of a triglyceride is inversely proportional to the saponification index (Moretto and Fett, 2008). The oil of the seeds of the two melon cultivars presented a mean molecular mass of 893.33 g mol⁻¹ for the oil of the *Pele-de-sapo* Melon seeds and 902.33 g mol⁻¹ for the oil of the *Pele-de-sapo* Melon seeds. The values found show that the lower the value found for the average molecular mass of the vegetable oils of the larger melon seeds was the saponification index.

Conclusion

The oil *Valenciano* Melon seeds and *Pele-de-sapo* Melon seeds presented good values of 28.47% and 26.41% respectively, appearing as an alternative source for utilization of two agroindustrial rejects.

The analysis of the composition of the major fatty acids present in the oil of the *Valenciano* Melon and *Pele-de-sapo* Melon seeds demonstrates the presence of Palmitic, Arachidonic, Linoleic (ω -6), Oleic (ω -9), Linolenic (ω -3) and Behenic.

The thermogravimetric analysis showed, through the graphs obtained by TG/DTG, the thermal behavior of the oils of the melon seeds, evaluating their stability, being this a determinant factor in the quality control, besides characterizing its viability of degradation through temperature and industrial use.

The quality parameters of the seeds oils of the two melon cultivars showed that they have a good state of conservation, since their quality parameters point to a possible use in human consumption, being able to be used in the food, pharmaceutical and cosmetic industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analytical quantification of copper in frogs (*Rana tigrina*) found from various aquatic habitats

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This study focused on the quantitative determination of Cu metal in various physical parts of frogs by using the Atomic Absorption Spectrometry technique). Cu was measured ($2.72 \pm 0.77 \mu\text{g/g}$) in kidney of *Rana tigrina* captured from industrial site and in *R. tigrina* captured from non-industrial site ($28.10 \pm 27.02 \mu\text{g/g}$). Similarly, $27.56 \pm 9.92 \mu\text{g/g}$ Cu was discovered in liver of *R. tigrina* captured from industrial site and $6.56 \pm 3.06 \mu\text{g/g}$ in *R. tigrina* captured from non-industrial site. From the results of the present study, it may be concluded that frogs are good bio-indicator species to estimate the level of heavy metals pollution of various water sources in some countries.

Key words: Copper, bio-indicator, atomic absorption spectrometry (AAS), aquatic habitat.

INTRODUCTION

Heavy metals are referred to as those elements which have relatively high density and are suspected to be toxic and very much toxic to the animals, humans, plants and aquatic lives at minute concentration levels (Singh, 2005). Amongst these heavy metals, approximately 30 elements are considered to be harmful to humans. Out of these elements, some are crucial for normal functioning of living cells, but they turn into lethal when taken above their recommended cut off limits. However, others can be xenobiotic, yet a bunch of them is very much toxic (Fraústo da Silva and Williams, 1993; Tamás and Martinoia, 2005). In addition, some of these metals are

essential for some creatures and are not vital for others in which they may have toxic effects (borderline). Since the requirements of living organisms are concerned, metals and metalloids are distinguished into three classes: essentials, nonessentials and borderline class. Usually, heavy metals are considered non-biodegradable and are buffered to environmental effects for prolonged times and ultimately cause serious pollution tribulations. Some of these harmful elements get access to molecular targets by means of food chain and by respiratory system and subsequently may possibly accumulate in organisms and causing hazards in the long run (Scheiffler et al., 2006).

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So, after bioaccumulation, heavy metals become perilous to organisms and their bioaccumulation depends upon their amount taken, their bioavailability, route adopted for their intake and their storage as well as excretion processes. Bioaccumulation of such elements in living organisms is faster than their catabolism at any time (Chapman et al., 1996). Potential toxicity in living organisms caused by exposure to high metal concentrations may follow various kinds of mechanisms. Proteins which have the ability to truss with metals play a critical role in this sense as they turn into a vehicle sources for the cellular metal toxicity (Thirumoorthy et al., 2011; Chiarelli and Roccheri, 2016). Both positive and negative effects on human health and environment are alleged from trace elements. Potentially toxic heavy metals may include Cd, Pb, Hg, Al, As, etc. and essential elements may include Fe, Mn, Cu, Zn and Se (Munoz-Olivas et al., 2001; Jalbani et al., 2007).

Potentially toxic elements are much dangerous when ingested even at very low concentration particularly because of prolonged exposure. The essential elements may also pose hazardous effects when taken beyond their recommended intake (Celik et al., 2007; Pouretdal and Rafat, 2007; Tao et al., 2012; Krebs et al., 2014; Hrabeta et al., 2016). Industrial effluents and agricultural drains are the main causes for the heavy metal contamination of sea water and direct disposal of sewage on waterfront enhances this contamination. Water treatment plants are rarely implanted to avoid this contamination. This heavy metal pollution badly affects the quality of sea water which makes it harmful for biota and human being via sea food chain. Quantitative determination of heavy metal contents in the entire body and tissue levels of marine organisms mostly concerns mollusks and crustaceans, but evidences have also been reported for coelenterates, polychaetes, and echinoderms. However, some aquatic organisms have ability to regulate internal metal levels. For example, mussels have this ability. Oysters have also been reported to have this ability. But instead of having similar feeding channels, former species have more effective abilities than later ones (Reidel et al., 1995; Tyokumbur and Okorie, 2011). In most mining areas, surface water and ground water are usually contaminated and polluted by heavy metals. Sources of the heavy metals in waters can either be natural (geogenic) or anthropogenic (Wong et al., 2003; Aidaikpoh et al., 2005; Akoto et al., 2008). Mining and smelting plants are the main anthropogenic sources of heavy metal contaminations in any mining area. The heavy metal contaminations are important due to their potential toxicity for human being and the environments (Lee et al., 2007; Vinodhini and Narayanan, 2008; Nasir et al., 2017).

This research work was conducted to use frogs found from various aquatic habitats to access heavy metal contamination of various water sources. For this purpose, liver and kidney of frogs were evaluated, as these organs

are suspected to bio-accumulate heavy metals in them. Hence, frogs were found to be good bio-indicators of heavy metal contamination of various water sources.

MATERIALS AND METHODS

Samples collection

Three aquatic habitats sewage, fish pond and canal water were selected to collect frogs. A total of 75 frogs were captured from these habitats during August to September 2012 at 40°C. All the sample collection areas were non-industrial. Twenty five frogs were captured from each site. Canal water samples of frogs were captured from Nawabpur canal situated in Multan city, Pakistan. Five equidistant sites of canal were used to get five frogs from each site. Sewage water samples of frogs were captured from the effluents of officer residence colony, Bahauddin Zakariya University, Multan, Pakistan. Similar pattern of almost equidistant five sites was taken in consideration and fish pond water samples of frogs were captured from five various fish ponds which were located in the area of Matti Tal Road, near Multan City, Pakistan (Figure 1). Five frogs were taken from each pond. All samples were weighed and preserved properly till sample preparations.

Samples preparation

Frogs were dissected and liver and kidney tissues were obtained from all the samples. After weighing, tissue samples were preserved in 70% ethyl alcohol solution and all these samples were stored at -20°C. The solution of tissue samples was prepared using aqua regia. Each tissue sample (0.1 to 0.2 g) was mixed to a volume of 3 ml of aqua regia and then this volume was subjected to reflux for half an hour at 150°C. Later on, this solution was cooled to room temperature and then 10 ml volume of deionized water was added to each sample. These solutions were further filtered by using Whatman No. 42 filter papers. All samples were further diluted after filtration by adding 25 ml of deionized water and these samples were stored at room temperature before analysis.

Standard preparation

A standard solution of copper (heavy metal) was prepared by using 0.1000 g of Cu wire in a final volume of 100 ml of nitric acid (corresponding to a concentration was 1000 ppm). Various working standard solutions of Cu were prepared by using dilution formula $N_1V_1=N_2V_2$. These working standard solutions were of the concentrations (100, 10, 0.3, 0.5, 1, 1.5, and 2 ppm) and these standards were properly tagged.

Copper analysis

An instrument of Atomic Absorption Spectrometer model A-1800 by Hitachi, Japan, was used for the quantitative analysis of Cu metal in various samples. Before starting the analysis, the instrument of Atomic Absorption was warmed up for 30 min. To ensure the correct measurements, a blank was always run for each sample prior to its analysis. For the purpose of calibration and to evaluate the performance of the described instrument, working standard solutions were run on the instrument. Later on, the solutions of sample were aspirated to measure their absorbance. Blank runs were used to ensure cross contamination and interferences. The quantitative analysis of analyte was done using calibration curves and statistics were completed on spreadsheets. The optimum

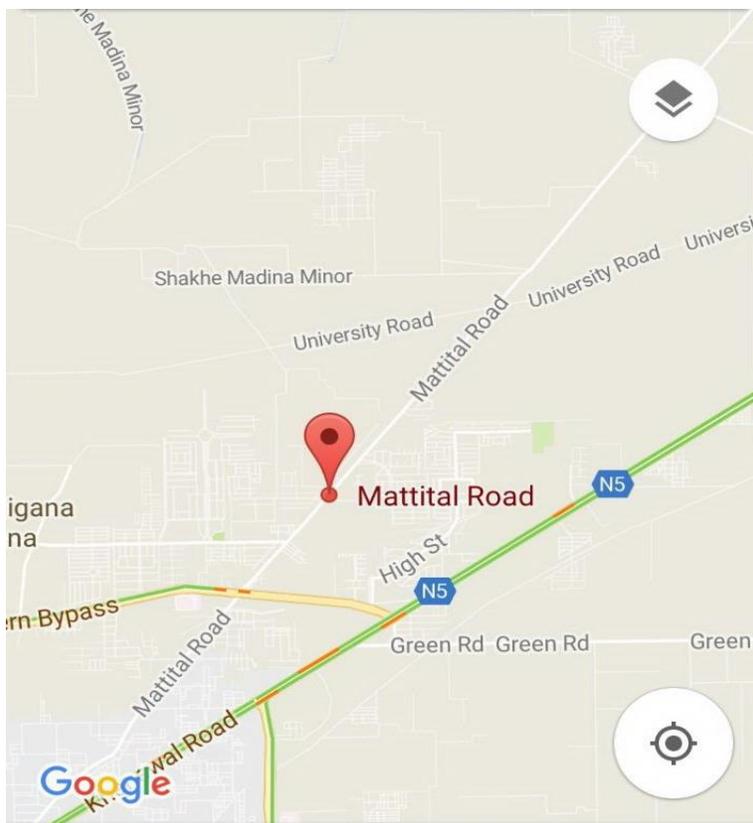


Figure 1. The study area (Mattital Road, Multan, Punjab, Pakistan).

conditions of the Atomic Absorption instrument were: lamp current 7.5 mA, slit width 1.3 nm, burner height was 7.5 mm, pressure of fuel was 0.30 kg/cm^3 , λ_{max} found for Cu was 324.8 nm and limit of detection (LOD) was found to be 0.04 mg/L. Flame composition consisted of the mixture of C_2H_2 and air. Calibration range used was 0.3 to 5.0 mg/L.

RESULTS AND DISCUSSION

In the present study, various tissues, for example kidney and liver, of frogs were analyzed to quantify Cu concentrations in them, as the ultimate aim was to govern the heavy metal contamination level of various aquatic habitats. All frog tissue samples and standards were analyzed in triplicate. Cu concentrations in the samples are summarized in Table 1.

The results of Cu metal concentrations in liver tissues of frogs collected from the canal water, fish pond and sewage waters compared with the results of Cu metal concentrations in kidney tissues of frogs collected from the same habitats have been summarized in Table 1. The accumulation of Cu in liver tissues of frogs from different habitats is found to be greater as compared to kidney tissue. The range of total body weight of frogs found in canal water was recorded as 54.26 to 98.49 g and mean was 71.27 ± 10.69 g as shown in Table 1 whereas range

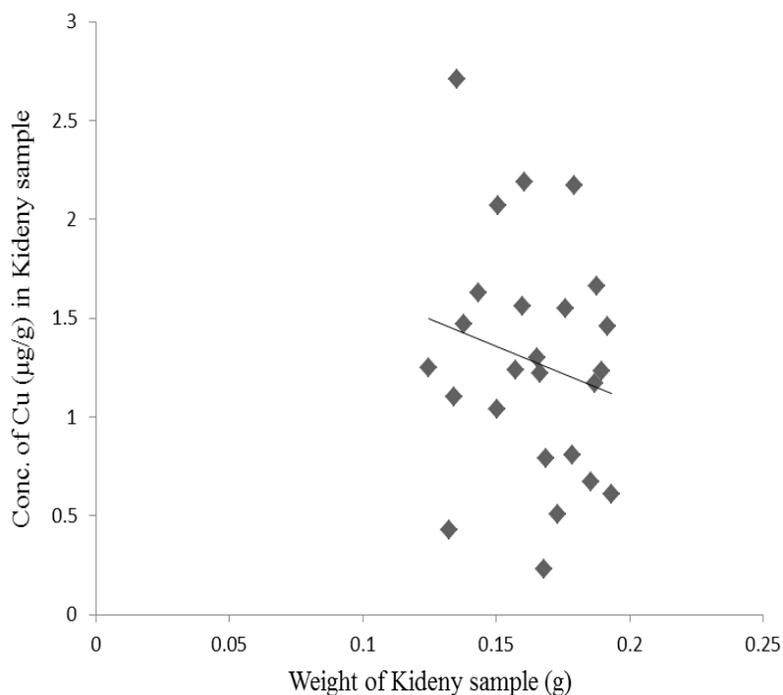
of total body weight of frogs found in fish pond water was recorded as 57.20 to 67.30 g and the mean was 60.80 ± 02.60 g as described in Table 1. The range of total body weight of frogs found in sewage water was 69.30 to 89.50 g, and the mean was 78.56 ± 06.20 g as described in Table 1.

The range of total kidney weight of frogs found in canal water was 0.13 to 0.19 g, mean is 0.16 ± 0.02 g as described in Table 1. The range of total kidney weight of frogs found in fish pond water was 0.12 to 0.20 g and the mean was 0.16 ± 0.03 g as described in Table 1.

The range of total kidney weight of frogs found in sewage water was 0.11 to 0.20 g, mean is 0.16 ± 0.03 g as described in Table 1. The range of Cu concentration ($\mu\text{g/g}$) in kidney of frogs found in canal water was 0.23 to $2.71 \mu\text{g/g}$, and mean was $1.28 \pm 0.60 \mu\text{g/g}$ as described in Table 1. The range of Cu concentration ($\mu\text{g/g}$) in kidney of frogs found in sewage water was 0.09 to $3.99 \mu\text{g/g}$, and mean was $1.54 \pm 1.10 \mu\text{g/g}$ as described in Table 1. The frogs found in sewage water have greater Cu concentration in kidney than frogs found in canal water as described in Table 1. It is clear from Figures 2 and 3, that as the weight of the kidney increased the accumulation of Cu decreased. As the kidney and total body weight of frogs increased the accumulation of Cu decreased in the kidney. It means that there is an inverse relationship

Table 1. Statistical data for different parameters of frogs taken from different habitats.

S/N	Location	Parameter	Range	Mean
1	Canal water	Total body weight (g)	54.26-98.49	71.27±10.69
2	Fish pond water	Total body weight (g)	57.20-67.30	60.80±02.60
3	Sewage water	Total body weight (g)	69.30-89.50	78.56±06.20
4	Canal water	Kidney weight (g)	0.13-0.19	0.16±0.02
5	Fish pond water	Kidney weight (g)	0.12-0.20	0.16±0.03
6	Sewage water	Kidney weight (g)	0.11-0.20	0.16±0.03
7	Canal water	Cu conc. (µg/g) in kidney	0.23-2.71	1.28±0.60
8	Sewage water	Cu conc. (µg/g) in kidney	0.09-3.99	1.54±1.10
9	Canal water	Liver weight (g)	0.13-0.19	0.16±0.02
10	Fish pond water	Liver weight (g)	0.12-0.20	0.16±0.026
11	Sewage water	Liver weight (g)	0.13-0.20	0.16±0.03
12	Canal water	Cu conc. (µg/g) in liver	4.27-46.22	20.51±7.32
13	Fish pond water	Cu conc. (µg/g) in liver	3.38-20.05	11.77±4.99
14	Sewage water	Cu conc. (µg/g) in liver	3.22-28.68	19.00±5.30

**Figure 2.** Effect of kidney weight (g) of frogs on the concentration of copper found in canal water habitat.

between kidney, total body weight of frogs and the Cu concentration in kidney. However, Cu concentrations are not quantifiable in kidney tissues of *R. tigrina* from fish pond water. It may be concluded that the frogs of low weight or small size may get their food from the soil having Cu. As the weight or size of the frogs increases they get their food from the insects or from dissolved organic or inorganic matter in water. It can be concluded

from the study that the kidney is the main organ for heavy metals accumulation. *R. tigrina* shows considerable large amount of Cu. It was slightly high in the liver as compared to other tissues. Kidney is the main organ of heavy metal accumulation in fishes (Hogstrand and Haux, 1991), amphibians (Suzuki and Kawamura, 1984), and mammals (Torra et al., 1994). Metals can enter through the water-permeable skin and the gut and then,

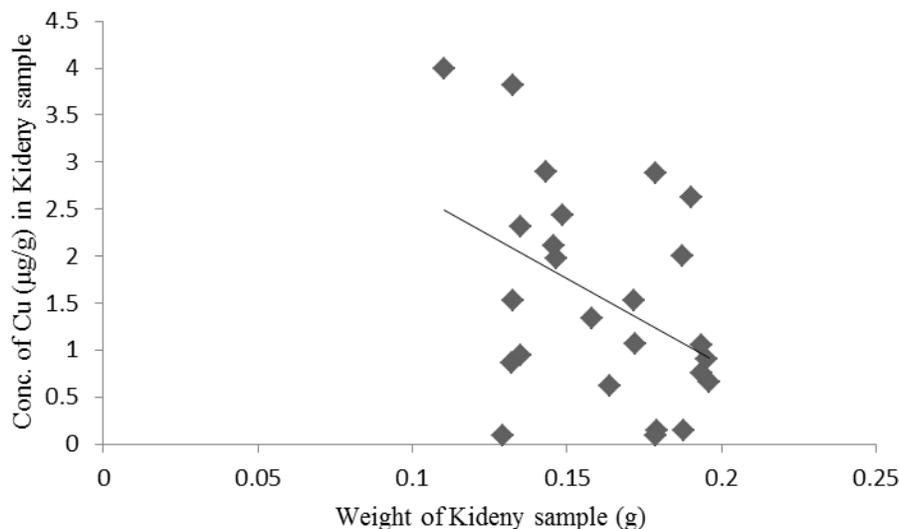


Figure 3. Effect of kidney weight (g) of frogs on the concentration of copper found in sewage water habitat.

via the blood circulation, accumulate in the liver and other tissues (Papadimitriou and Loumbourdis, 2003).

The weight of liver of frogs that live in canal water ranged from 0.13 to 0.19 g and the mean was 0.16 ± 0.02 g as described in Table 1. The weight of liver of frogs that live in fish pond water ranged from 0.12 to 0.20 g; mean is 0.16 ± 0.026 g as described in Table 1. The weight of liver of frogs that live in sewage water ranged from 0.13 to 0.20 g, and the mean was 0.16 ± 0.03 g. The frogs found in canal water have greater average weight as 0.1638 g, than frogs found in fish pond and sewage water. The range of Cu concentration ($\mu\text{g/g}$) in liver of frogs that live in canal water was 4.27 to 46.22 $\mu\text{g/g}$, and the mean was 20.51 ± 7.32 $\mu\text{g/g}$ as described in Table 1. The range of Cu concentration ($\mu\text{g/g}$) in liver of frogs that live in fish pond water was 3.38 to 20.05 $\mu\text{g/g}$, and the mean was 11.77 ± 4.99 $\mu\text{g/g}$ as described in Table 1. The range of Cu concentration ($\mu\text{g/g}$) in liver of frogs that live in sewage water was 3.22 to 28.68 $\mu\text{g/g}$, and the mean was 19.00 ± 5.30 $\mu\text{g/g}$ as described in Table 1. The frogs found in canal water had greater Cu concentration in liver than frogs found in fish pond and sewage water. The frogs that lived in sewage water had Cu concentration in liver greater than frogs found in fish pond water. As shown in Figures 4, 5 and 6, as the weight of liver increased, the Cu concentration accumulation in liver decreased. It means that there was an inverse relationship between liver weight of frogs and the Cu concentration. It may be concluded that the frogs of low weight or small size may get their food from the soil having Cu. As the total body weight or size of the frogs increases the concentration of accumulation of Cu in liver increases. A comparative study of the results of bioaccumulation of Cu metal concentrations ($\mu\text{g/g}$) in liver and kidney tissues of frogs collected from the canal

water, fish pond and sewage waters indicate that Cu levels in liver tissues are comparatively higher than in kidney tissues. However, Cu concentrations are not quantifiable in kidney tissues of frogs from fish pond water.

The liver is the chief organ for metal homeostasis, being the site of metalloenzyme production and metal storage as well as excretion via the bile duct. The liver also has the highest metal load compared with other tissues, and so it reveals the bioavailability of metal concentrations in the water bodies (Stolyar et al., 2008). Accumulation of heavy metals affects the metabolic activities resulting in a decrease in body length and body weight (Stolyar et al., 2008). Toxic concentrations of heavy metals increase the biochemical stress in the organisms due to deterioration of metabolic cascades (Hudecova and Ginter, 1992). Balance between production and catabolism of the oxidants is important to maintain biological functioning of organisms. Metabolism activities, however, varies with temperature changes. Frogs are poikilothermic vertebrates, and heat stress can affect their bodily metabolism. Relatively greater concentrations of Cu in the liver were recorded as compared to the kidney in *R. tigrina*. *R. tigrina* showed higher concentration of Cu in the liver. Similarly, Loumbourdis and Wray (1998) also reported greater concentration of Cu in the liver as compared to other organs and highlighted that frog accumulates higher Cu indicating its role in detoxification and storage. Measured higher concentration of Cu could be related to detoxification and storage mechanisms. Liver tissue accumulated higher mean concentrations of heavy metals in comparison to the other body tissues. The liver is a major detoxifying organ of the body, and besides this, it is the main center where hemoglobin breakdown

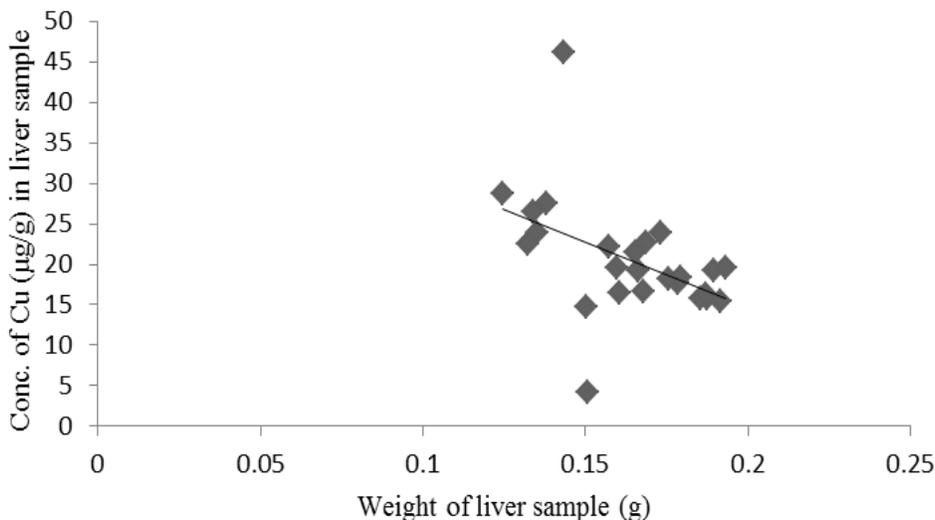


Figure 4. Effect of liver weight (g) of frogs on the concentration of copper found in canal water habitat.

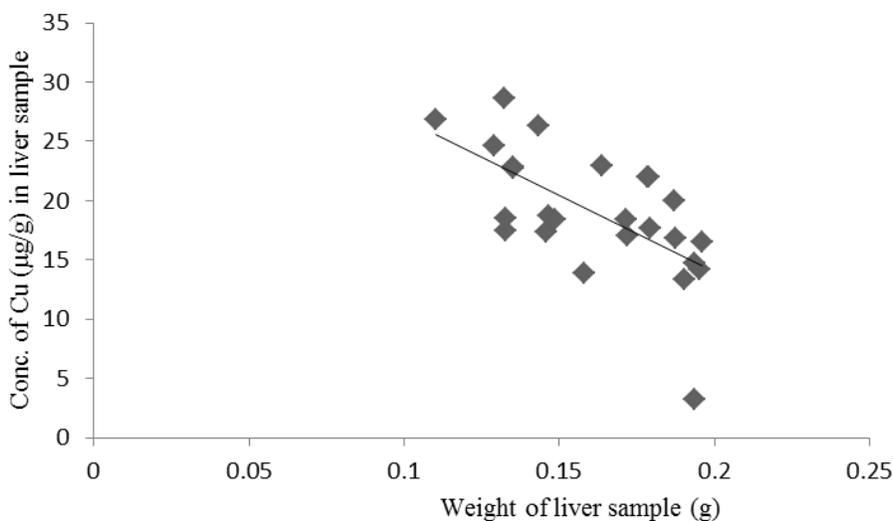


Figure 5. Effect of liver weight (g) of frogs on the concentration of copper found in fish pond water habitat.

occurs. Cu concentration also turned very high which is probably due to the presence of Cu in the compounds like fungicides and insecticides abundantly used in agriculture practices carried out in the study area. Higher values of Cu in the liver may be due to its detoxification by virtue of metallothionein and other metal-binding proteins (Lance et al., 1995). The uptake and removal patterns of metals depend on multiple factors that scale with body size including surface area to volume ratios. This further suggests *R. tigrina* to be a good bioindicator of water contaminants like the heavy metals (Qureshi et al., 2015).

Conclusion

The present study is relevant to human health because heavy metals are produced by different industries like tanneries for leather processing and added into water reservoirs as a waste. This water enters the food chain of human beings. The heavy metals cause physiological abnormalities. Outcomes of the present study showed that liver tissues of frogs taken from all the three habitats (canal water, fish pond and sewage water) contain higher level of Cu contents than kidney tissues. There is an inverse relation between kidney weight and Cu contents

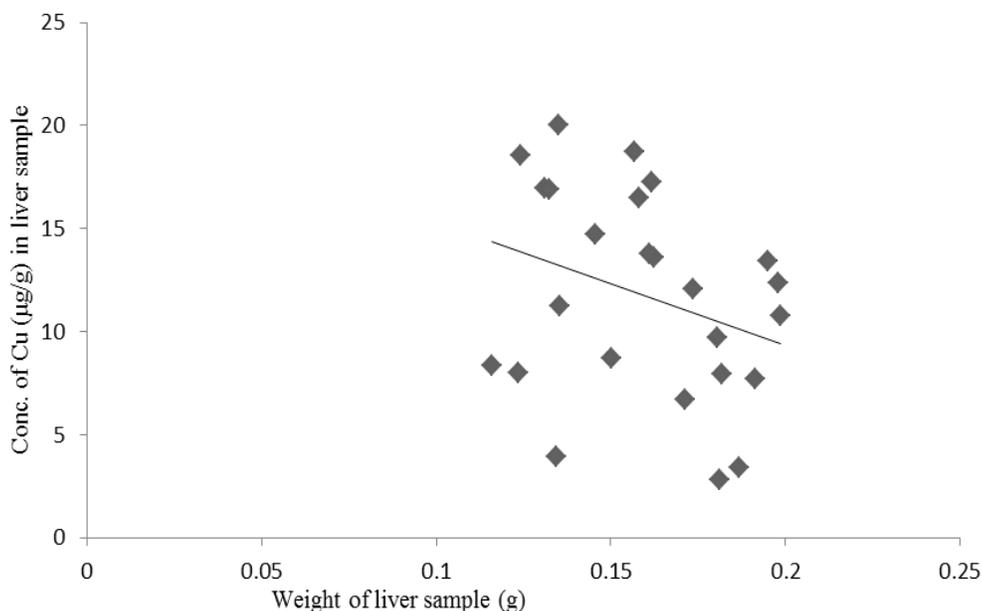


Figure 6. Effect of liver weight (g) of frogs on the concentration of copper found in sewage water habitat.

in it. As kidney weight increases the concentration of Cu decreases. Direct relation has been found between total body weight and Cu contents in kidney. As total body weight of frogs increased the accumulation of Cu increases in kidneys of frogs. Inverse relation has been found between liver weight and Cu contents. As the weight of liver increased, the Cu concentration in liver decreased. A direct relation exists between total body weight of frogs and Cu contents in liver, as the body weight increased the accumulation of Cu in liver increases. Frogs are good biological indicator to assess the heavy metals contamination in aquatic environments.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Immobilization of the Carioca bean (*Phaseolus vulgaris* L.) extract on SiO₂-TiO₂ aiming the determination of the herbicide Simazine

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Pesticides are identified as hazardous contaminants due to high toxicity and persistence in the environment. In this context, it is necessary to monitor these pollutants in the environment *in situ*, with the use of low cost analytical techniques and of quick response. Biosensors are presented as a complementary analytical tool to more complex techniques, such as chromatography. The present work aims to develop, in a simple and low cost way, an amperometric biosensor for the detection of simazine in aqueous samples from the inhibition of the enzyme peroxidase extracted from the pinto bean (*Phaseolus vulgaris* L.). For the development of the biosensor, the enzyme peroxidase was extracted in sodium phosphate buffer solution, of pH 6.0, and pre-purified by ammonium sulfate precipitation 40% (w / v) salt saturation to 50.0 ml of the protein extract. Peroxidase was characterized by electrophoresis and immobilized on silica-titanium oxide, synthesized by the sol-gel method in the proportions of 70% / 30% respectively. The biosensor showed linearity in the concentration range between 1.0 and 6.0 µg L⁻¹ of simazine (R² = 0.989) in the potential range 0.20 V to 0.30 V vs Ag / AgCl by the square wave voltammetry technique. The curve was obtained in the presence of 2.0 mg L⁻¹ phenol and 3% H₂O₂ (v / v), where the current density signal decreased in the presence of successive additions of the simazine inhibitor. The biosensor was applied to a standard simazine-fortified sample and detected the presence of simazine at the concentration of 35.05 µg L⁻¹. Even showing a relative percentage error of 12.63%, when compared with the result of the analysis by high performance liquid chromatography, the biosensor is useful to monitor the presence or absence of the simazine pesticide in the solution.

Key words: Simazine, Biosensor, Carioca beans (*Phaseolus vulgaris* L.)

INTRODUCTION

Simazine (2,4-Bis(ethylamino)-6-chloro-1,3,5-triazine) is a selective herbicide for weeds grouped into the triazine

class (Guo et al., 2014). It constitutes several formulations of pesticides applied mainly in corn, beans, sugarcane and soybean (Morgante et al., 2012; Keren et al., 2015). The continuous and excessive use of this pollutant severely damages soil and water bodies (Gunasekara et al., 2007) due to its toxicity and persistence time in the environment (Parte et al., 2017; Odukkathil and Vasudevan, 2013), in addition to offering a risk to people exposed to this pesticide (Alavanja et al., 2013). Studies report that Simazine can trigger allergies and diseases such as cancer (Sato et al., 1998; Scognamiglio et al., 2013). Monitoring of agricultural pesticides has been studied in the field of development of amperometric biosensors, since these are devices that have high sensitivity and specificity, with the advantages of miniaturization (Mihos et al., 2014), easy transportation and reduced analysis time, being able to determine concentrations in scale of mgL^{-1} a μgL^{-1} (Zamora-Sequeira et al., 2019; Gil and De Melo, 2010). Enzymatic biosensors are assembled by immobilizing functionalized proteins that act specifically for a particular substrate (Yulaev et al., 2001; Campanella et al., 2011). Generally purified commercial enzymes are used in the assembly, which makes the device more expensive (Zamora-Sequeira et al., 2019). One of the proposition of this work is to assemble an enzymatic biosensor from the peroxidase enzyme extracted from the Carioca Bean (*Phaseolus vulgaris* L.) grains. This legume is a rich, low-cost protein peroxidase rich source that can be extracted and applied to detect herbicides such as simazine. The success of the herbicide detection is based on its interaction by inhibition over the peroxidase. In order to the bio-reaction to occur, the active peroxidase site must be available and free of deformations, so that a molecule of hydrogen peroxide (natural substrate) oxidizes the ferroporphyrin (Fe^{+3}) group and produces the $\text{Fe}^{+4}/\text{Fe}^{+5}$ highly unstable intermediates (Pérez Galende et al., 2015; Aisha et al., 2016; Jang and Moon, 2011). In this step the phenol in solution can act as an electron mediator, recovering the initial oxidation state (Fe^{+3} from the native enzyme) and repeating the cycle. The selectivity of peroxidase can be optimized by immobilization of the enzyme on a $\text{SiO}_2\text{-TiO}_2$ composite, which blocks the direct transfer of electrons from the electrode and makes it depend only on phenol as electron donor. From then on, the reduction current of the quinones, product obtained from phenol oxidation, can be evaluated at the solution-electrode interface (Morales et al., 1996).

The detection of the Simazine herbicide occurs by evaluation of the cathodic reduction current of the quinones before and after the contact with the inhibitor (Songa et al., 2009; Arduini and Amine, 2014).

EXPERIMENTAL

Extraction of peroxidase from beans of pinto bean (*Phaseolus vulgaris* L.)

Pinto bean (*P. vulgaris* L.) was purchased from EPAMIG (Minas Gerais-Brazil), lot FELPI 001. The grains were grounded in manual grinder. In the process the fractions of shell and pulp were separated using a 0.3 mm nylon sieve. The extracts were obtained by blender homogenization from 50.0 g of bean powder, 5.0 g of polyvinylpyrrolidone (PVP) in 150.0 ml of pH 6 sodium phosphate buffer 0.1 mol.L^{-1} for 5 min. The obtained homogenate was gauze filtered to remove larger fibers and the filtrate was subjected to centrifugation on a rotation of $13584 \times g$ at 2°C for 30 min. The extracts were stored at -20°C for further analysis.

Pre-purification of pinto bean extract

In a 0°C ice bath system, a 50.0 ml aliquot of crude enzymatic extract was subjected to precipitation in ammonium sulfate 40% (w/v). After precipitation, the sample was centrifuged at $13584 \times g$ under 4°C refrigeration for 20 min. The protein precipitate was resuspended in 5.0 ml pH 6 sodium phosphate buffer. After concentration, the extract was dialyzed using 12 kDa pore aperture cellulose semipermeable membrane versus pH 6 buffer solution. The procedure was performed for 12 h in a refrigerated chamber at 4°C .

Characterization of peroxidase extracted by SDS-PAGE

The peroxidase present in pinto beans was characterized by electrophoresis under denaturing conditions (SDS-PAGE), as described in the literature (Laemmli 1970), with modifications. For the resolution gel were added 0.8 ml of Milli-Q water, 4.125 ml of acrylamide/bis-acrylamide solution (30: 0.8), 5.0 ml of Tris-HCl-SDS $0.75 \text{ M/pH } 8.8$ buffer, 75 μl of 10% (w/v) APS (ammonium persulfate) and 5.0 μl TEMED(N,N,N',N'-tetramethylethylenediamine). The application gel was prepared by mixing 1.925 ml of Milli-Q water, 0.5 ml of acrylamide/bisacrylamide solution (30:0.8), 2.5 ml of $0.25 \text{ M/pH } 6.8$ Tris-HCl-SDS Buffer, 75.0 μl of 10% (w/v) APS and 7.5 μl of TEMED. Protein denaturation was achieved by mixing 20.0 μl of crude bean extract and 20 μl of 0.6 mol.L^{-1} pH 6.8 Tris-HCl buffer in the presence of β -mercaptoethanol. The sample was heated in a water bath at 100°C for 5 min. The running conditions were 200 V, 60 mV and 70 W, lasting 45 min. The gel was stained with 0.01 M silver nitrate solution in the presence of alkaline solution containing chloroform. After revealing the protein bands, the gel was photographed and the digital image analyzed by GelAnalyzer 2010 software.

Determination of total protein content

The total protein content of the bean extract was determined according to the method described by Bradford (1976). The readings were performed in UV-Visible spectrophotometer at 595 nm wavelength. The obtained values were compared with the standard curve of bovine serum albumin (BSA) in concentrations of 0.10 to 1.40 mg.ml^{-1} .

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Determination of peroxidase activity

The enzymatic activity of peroxidase was measured following the procedure of Queiroz et al., (2007). The extracts were diluted to 1:10 in 0.1 mol.L⁻¹ pH 6 sodium phosphate buffer. In three test tubes, 500 µl of enzyme extract, 500 µl of 3% (v/v) hydrogen peroxide and 2.0 ml guaiacol 2.5 mg/100 ml were added and incubated in a water bath for 3 min. The reagent mixture was homogenized and immediately poured into quartz cuvette. The readings were performed on UV-visible (Shimadzu UV-1800) spectrometer, at 470 nm wavelength, at intervals of 1 s, for 300 s. The activity was investigated, in triplicate, by monitoring the tetraguaiacol formed in the enzymatic reaction. An enzyme unit was defined as the amount of enzyme that caused the increase of 0.001 unit of absorbance per minute of reaction.

Synthesis of SiO₂-TiO₂ oxide

The silica-titanium oxide was synthesized by the sol-gel method, according to the procedure described in Oliveira (2012). Proportions were prepared for synthesis of the material with 70% SiO₂ and 30% TiO₂. To a round bottom flask it was added 133.0 ml of tetraethylorthosilicate (TEOS 98%) along with 133.0 ml of anhydrous ethanol. After homogenization, 11.00 ml of 3.5 mol.L⁻¹ HCl was added in order to promote the prehydrolysis of tetraethylorthosilicate. The solution was left under oil bath at 60°C for 3 h with stirring. After this time, 66.00 ml of titanium butoxide (ButOTi) and 11.0 ml of 3.5 mol.L⁻¹ HCl were added and left for a period of 20 h at 60°C. At the end of this step the material was transferred to a becker and kept in oven at 60°C for a period of three days for complete evaporation of solvent residues still present in the medium. After drying, the material was deagglomerated with the aid of a glass stick and subjected to vacuum (10-3 mmHg) for removal of residual solvent. The samples were prepared in KBr pellets and analyzed by NICOLET Magna-IR 760 spectrophotometer

Immobilization of peroxidase in SiO₂-TiO₂ oxide

The peroxidase enzyme was immobilized on silica-titanium oxide by homogenizing 0.125 g of SiO₂-TiO₂, 1.0 ml of pre-purified extract containing 2500 U/ml and 100 µl of 0.5% (v/v) pH 6.0 glutaraldehyde on a petri dish. The mix was allowed to dry at room temperature (25°C) for 30 min. After drying, the oxide was removed with the aid of a spatula and the obtained powder containing peroxidase enzyme was added to the carbon paste.

Scanning Electron Microscopy (SEM)

The morphological aspect of the carbon paste modified with SiO₂-TiO₂ was obtained using the scanning electron microscopy technique, using JEOL model JSM 6360-LV with 20 kV and 1000 x magnification. The mapping of the Si, Ti and O elements was carried out in a semi-qualitative way, by electron dispersive spectroscopy (EDS) coupled to the SEM. For analysis the samples immobilized on conductive double-sided tape and covered with gold.

Preparation of the graphite-SiO₂-TiO₂-Peroxidase paste

The carbon paste was previously prepared by the addition of 0.2934 g of graphite powder and 0.0754 g of mineral oil. With aid of porcelain mortar and pestle the mixture was homogenized for 5 min.

Finally, 0.125 g of the silica-titanium oxide containing the immobilized enzyme was added.

Electroanalytical system for biosensor use (working electrode)

The modified carbon paste (graphite-SiO₂-TiO₂-peroxidase) was added to the 0.4239 cm² area of the working electrode containing a copper wire. The system was composed by Ag/AgCl reference electrode (KCl_{sat}), platinum auxiliary electrode and working electrode. The amperometric determinations were obtained using a potentiostat/galvanostat (AutoLab 128n) and square wave voltammetry technique. The potential range studied was -0.20 to +0.50 V vs Ag/AgCl, frequency of 100 Hz, and amplitude of 0.005 V. The current measurements were obtained after 2 min of biosensor/solution contact. The electrolytic solutions without addition of simazine were prepared in phenol concentration 2.0 mg.L⁻¹ and 3.0% (v/v) hydrogen peroxide in pH 7 phosphate buffer. For each current acquisition the modified carbon paste was removed and new paste was added to the electrode.

Inhibition curve

The enzymatic inhibition curve was constructed from 0.5-10.0 µg.L⁻¹ of simazine in 2.0 mg.L⁻¹ phenol solution at pH 7 and 1.0 ml of 3.0% (v/v). Each point of the curve was obtained by recharging the working electrode with the modified carbon paste containing the enzyme. The cathodic current measurements for the presence of quinine were determined by square wave voltammetry. The data were collected after 2 min of contact between the working electrode and the substrate solution. After application of the working potential, the biosensor presented a current response after 12 s.

RESULTS

Characterization of pinto bean extract

The peroxidase extracted from carioca beans (*P. vulgaris* L.) showed total activity of 2500 U/ml, with a protein content of 87.37 mg/ml, which characterizes a specific activity of 66.02 U.mg⁻¹. The bean was characterized by electrophoresis as shown in Figure 1. The molecular mass of the commercial peroxidase is presented in literature as around 40 kDa (Sigma Aldrich 2017), while the peroxidase extracted from the bean (*P. vulgaris* L.) was identified at approximately 41.68 kDa.

Characterization of carbon paste and silica-titanium oxide

Medium Infrared (FTIR-MID)

After oxide synthesis, the pulverized sample (<90 µm) was analyzed by medium infrared (FTIR-MID) in KBr paste. The main bands for characterization of silica-titanium oxide are presented in the spectrum of Figure 2. The vibrational frequencies of SiO₂ can be identified by wide band at 3448 cm⁻¹ corresponding to the symmetrical

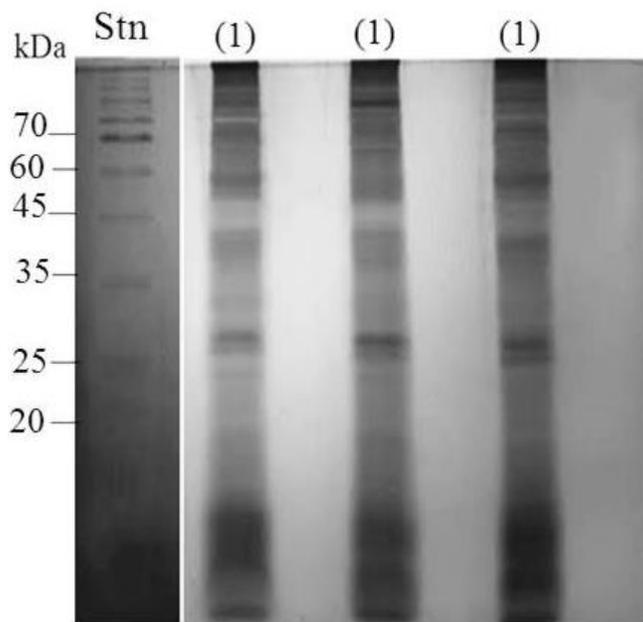


Figure 1. Gel 12.5% (SDS-PAGE) for characterization of peroxidase enzyme extracted from Pinto Beans. (Stn) True Color protein standard high range protein marker (Bio-Rad). (1) Pinto Bean Extract after pre-purification.

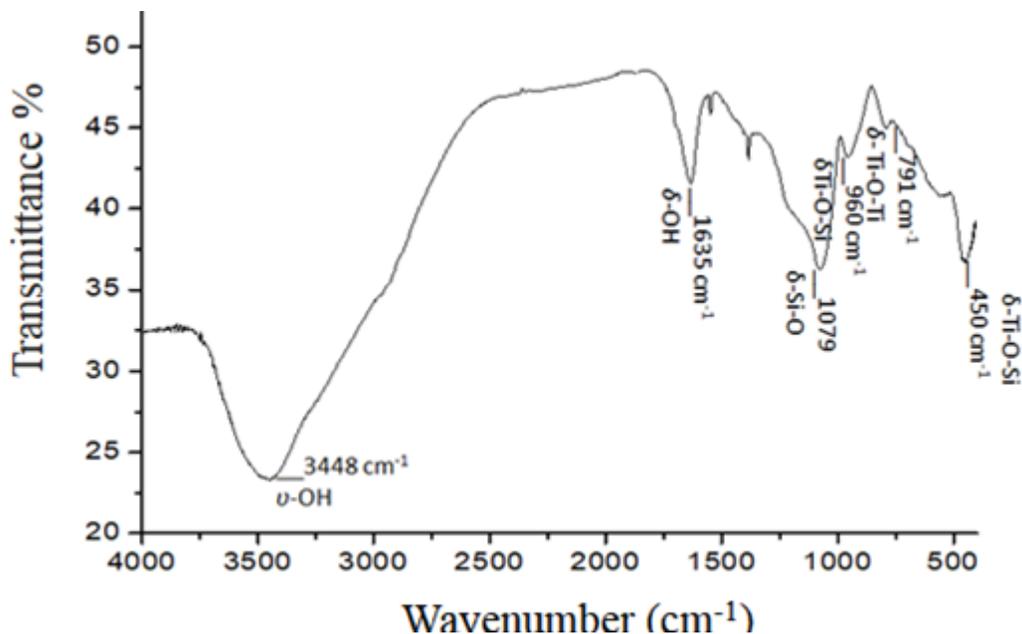


Figure 2. Spectrum in the medium infrared region for the oxide Silica-titanium used to immobilize the peroxidase enzyme extracted from Pinto Beans

stretching vibrations of the silanols (SiO-H) groups. The absorption at 1079 cm⁻¹ is due to axial vibrations of the Si-O group (Navarrete et al., 1996). The presence of titanium was observed by the vibrations at 960 cm⁻¹ for

the asymmetric stretching of the Ti-O-Si bond and the bands between 450 and 791 cm⁻¹ corresponding to Ti-O and Ti-O-Ti vibrations (Davis and Liu, 1997). By the absorption profile in the spectrum it can be inferred that

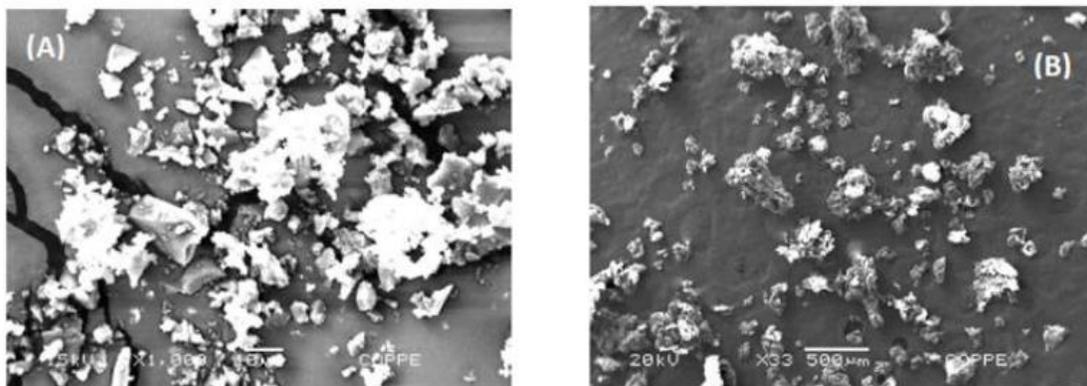


Figure 3. (A) Matrix surface for immobilizing the peroxidase enzyme, SiO₂-TiO₂ oxide with granulometry below 90 mesh, (increments of 1,000x). (B) Carbon paste modified with SiO₂-TiO₂ containing immobilized enzyme (increments of 33x).

the results obtained show that grafted TiO₂ are dispersed in the silica matrix.

Scanning electron microscopy (SEM)

The SiO₂-TiO₂ oxide prepared by sol-gel was reduced to particle size below 90 mesh and subjected to scanning electron microscopy (SEM) together with the modified carbon paste containing the enzyme, as shown in Figure 3A and B. As Figure 3A shows that the material exhibits diversity of sizes and shapes, which does not interfere on the purpose used. Also by the electron micrograph it is observed that the area selected for Energy-dispersive X-ray spectroscopy (EDS) analysis showed uniform distribution for SiO₂-TiO₂ by all material, and the formation of individual TiO₂ and/or SiO₂ agglomerates (islands) does not occur. According to microscopic scanning analysis (Figure 3B), it was possible to visualize the formation of enzyme-SiO₂TiO₂-Graphite agglomerates, which can provide a high concentration of active sites of the dispersed enzyme on the SiO₂-TiO₂ surface available for catalysis. In the stage of immobilization cross-links are formed between enzymes and glutaraldehyde and enzymes and support. Such bonds occur through terminal carboxylic and amine groups present in the molecular structure of the enzyme and the titanium oxide, grafted onto the surface of the silica. This immobilization was advantageous due to the increase of the enzyme-matrix contact surface with consequent increase in the rate of enzymatic catalysis.

Calibration curve for simazine

The percent inhibition of the immobilized peroxidase enzyme was determined from the cathodic current density values for quinone reduction. The inhibition curve

was constructed by increasing concentrations of simazine from 0.5 to 10 μg/L at pH 7.0. Current data were obtained at +0.14 V vs Ag/AgCl, in the absence and presence of the inhibitor (simazine). In the absence of the inhibitor, the biosensor had a maximum current density of -120.10 J/μAcm⁻², from the enzymatic catalysis of phenol oxidation (2.0 mg/L phenol and 3% H₂O₂ (v/v)). In the presence of the inhibitor, the cathodic current measurements were obtained after 2 min of contact with the solution containing simazine. The inhibition curve of peroxidase as a function of the concentration of simazine is shown in Figure 4. As shown in Figure 4 for established experimental conditions, the biosensor showed linearity in the concentration range 1.0 to 6.0 μg/L ($R^2 = 0.989\%$ inhibition = 1.073 [simazine] + 22.8) of Simazine. The modified carbon paste containing the peroxidase enzyme used to construct the calibration curve was also used to determine simazine in sample01.

Application of biosensor on samples

The herbicide Simazina has its commercialization controlled, regulated by law in several countries (Heri et al., 2008; Xie et al., 2019; Conama, 2005) in view of its high toxicity to humans and the environment. Thus, to evaluate the response of the biosensor a sample of Simazina was assigned by the Oswaldo Cruz Foundation (Fiocruz-Brazil) and analyzed by square wave voltammetry. For analysis using the biosensor the sample was diluted (10 times) in pH 7 phosphate buffer and applying scanning potential from -0.20 V to +0.50 V vs Ag/AgCl. The measurements were obtained in triplicate, from the same batch of modified carbon paste, as shown in Figure 5.

The currents were converted to current density, presenting values of -89.58, -88.24 and -86.74 J/μAcm⁻², with average current density of -88.19 J/μAcm⁻². The

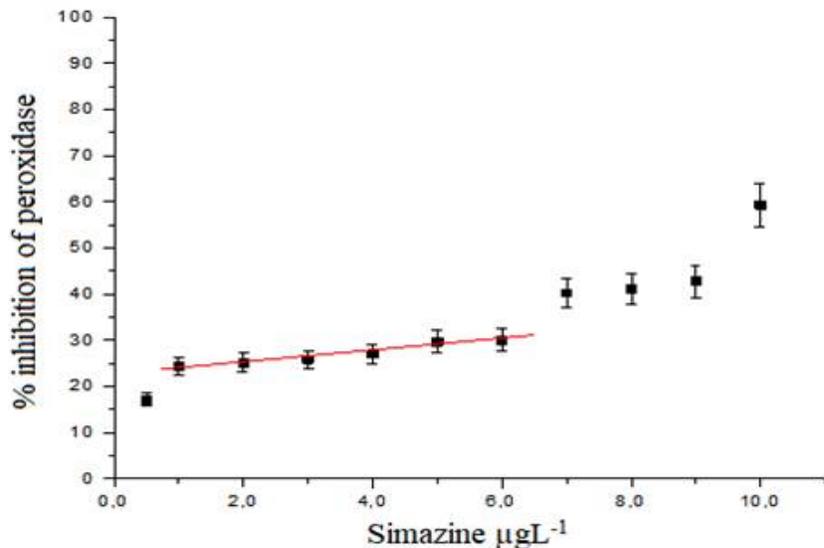


Figure 4. Inhibition curve of the peroxidase enzyme extracted from Pinto Beans (*P. vulgaris* L.) in the presence of the herbicide Simazine.

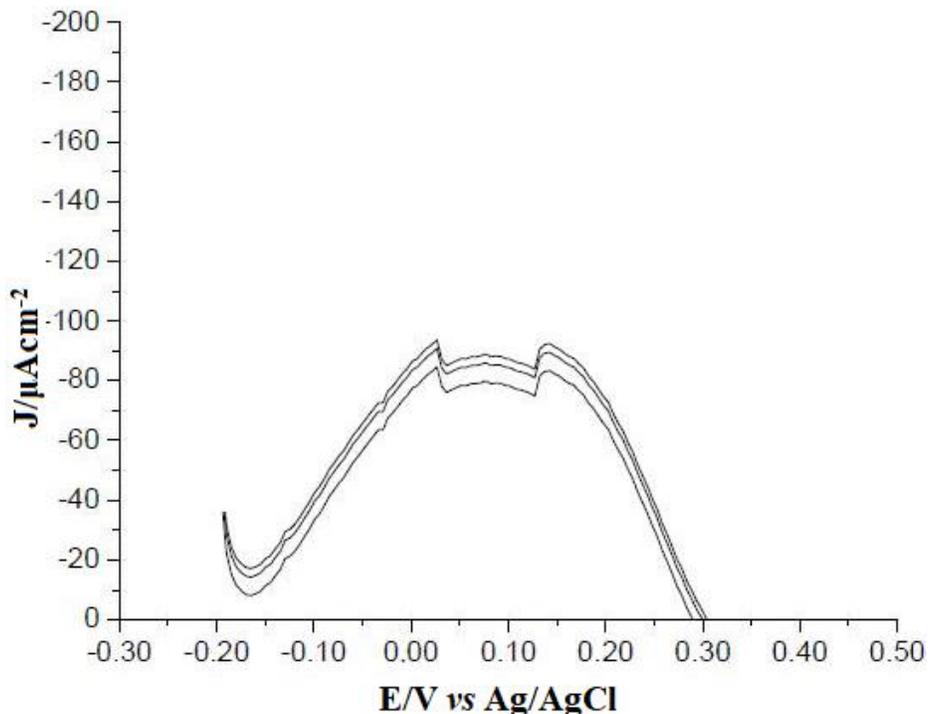


Figure 5. Biosensor response for triplicates of the sample containing Simazine. Current density vs potential electric using reference electrode silver / silver chloride

percentage of inhibition was obtained by applying the inhibition curve ($\%inhibition = 1.073 [simazine] + 22.8$). The inhibition percentage for the analyzed sample was 26.56%, corresponding to a simazine concentration of $35.04 \mu\text{g}\cdot\text{l}^{-1}$ in the sample (dilution factor: $FD = 10$). In

contrast, the sample was analyzed by high performance liquid chromatography (HPLC) from a calibration curve in concentrations of 0.02 to $0.032 \mu\text{g}\cdot\text{ml}^{-1}$.

The sample analyzed by HPLC showed a concentration of $31.11 \mu\text{g L}^{-1}$ Simazine ($R^2 = 0.997$). This result shows

that the proposed biosensor presented a relative percentage error of 12.63%, showing that the developed device does not present sufficient confidence in the detection of the herbicide. However, even with an error greater than 10%, the biosensor can be considered efficient for the verification of the presence or absence of simazine in aqueous samples. It is also a promising device considering the cost reduction analysis and manufacturing, compared to classical techniques such as HPLC, which demands time and high investment costs. The proposed biosensor also requires investigative studies regarding several variables that can be optimized to minimize detection errors, such as the storage time of the simazine solution after preparation and the time of contact between biosensor (peroxidase) and agrochemical simazine.

DISCUSSION

The results show that peroxidase present in the carioca bean extract, even if not totally purified, presented a good selectivity and specificity in relation to the catalytic route for phenol oxidation. This is also due to the fact that carbon paste based biosensors, which use electron mediators such as phenol, immobilizing peroxidase on the titanium silica matrix favours the biosensor selectivity for this molecule. This is due to the electron transfer blockage between the electrode (carbon paste) and the enzyme, which depends exclusively on phenol (electron donor), a fact that has already been observed by Rosatto et al., (1999). The decrease in the peroxidase phenol oxidation product reduction current signal suggests a mechanism of inhibition of the peroxidase active site, and further investigation is needed, which makes this research a basis for this investigation. There are studies based on immunosensors that use the peroxidase conjugation device with antibodies, in which the authors report that the inhibition is competitive (Yulaev et al., 2001).

Many enzymatic biosensors that use partially purified extracts showed good sensitivity measures and low percentual errors, while others had considerable detection errors above 10%. Moccelini et al., (2008) reports the construction of a biosensor from scarlet eggplant (*Solanum gilo*) extracts containing the peroxidase enzyme for phenolic compound detection, showing relative error of 4.5% and 1.5%, with detection limit for hydroquinone of $2.0 \times 10^{-5} \text{ molL}^{-1}$ and $3.0 \times 10^{-8} \text{ molL}^{-1}$ rutin.

Santos (2016) developed a biosensor from mushroom extract (*Agaricus bisporus*) partially purified with ammonium sulfate, containing the tyrosinase enzyme. The author determined the presence of benzoic acid in a guarana sample and obtained a percentual error above 10% in the samples when compared to HPLC measurements. This study reported the presence of

interferers in the media which caused errors in the determinations. The bean extract based biosensor containing peroxidase enzyme is a viable alternative of simple elaboration, providing linear response by the basic principles of amperometric detection. The good approximation of the results compared with HPLC shows that the device is efficient and selective and can be applied by the enzyme inhibition method in the presence of simazine pesticide, being able to validate the presence or absence of this contaminant in water. Improvement of the device is still necessary so that its performance can reach quantification levels and not just detection, with lower relative error for analysis. The mechanisms that control the inhibition reaction still needs to be investigated more clearly.

Conclusion

Pinto bean (*P. vulgaris* L.) is a rich source of peroxidase, low cost and easily accessible in several countries. The biosensor based on the extract of the bean grains containing the peroxidase enzyme is a viable alternative of simple elaboration, but with basic principles of amperometric detection, consolidated by several biosensors. Its use is potentialized in view of the need for environmental monitoring of contaminating pesticides on surface and groundwater sources, and it is possible to prepare the analysis in aqueous samples *in situ*. It was demonstrated that there is an inhibition response, by the decrease of cathodic current, when the biosensor comes in contact with the herbicide simazine, fact that shows the sensitivity of the device in the detection of this pesticide. The improvement of the device is still necessary so that its performance can reach levels of quantification and not only of qualification, with lower relative error for analysis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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