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Full Length Research Paper

Analysis of the genetic diversity of selected East African sweet potato (*Ipomea batatas* [L.] Lam.) accessions using microsatellite markers

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Sweet potato (*Ipomea batatas* [L.] Lam.) is an economically important crop in East Africa chiefly grown by small holder farmers. Sharing of vines for planting is a very common occurrence among these farmers and eventually varieties are given local names, making it hard to trace the original pedigree. It is therefore important to characterise the sweet potato germplasm for purposes of breeding and germplasm conservation. In this study, 68 sweet potato accessions were evaluated for diversity using 12 microsatellite markers. The genetic relationship of the germplasm was evaluated using the Jaccard's coefficient for dissimilarity analysis, unweighted pair group method with arithmetic means (UPGMA) tree and principal component analysis (PCoA) on DARwin software, while summary statistics was done using PowerMarker and Popgene softwares. The polymorphic information content of the markers ranged from 0.1046 for markers J67b and J67 to 0.3671 for marker J1809a, with a mean value of 0.2723. The total number of alleles amplified was 21. The major allele frequency ranged from 0.5882 for marker JB1809a to 0.9412 for markers J67b and J67c. Cluster analysis divided the accessions into four major clusters. Principle component analysis divided the accession into four groups which were different from those by cluster analysis. This study was able to identify several distinct accessions as well as a few possible duplicate accessions that overlapped on the cluster analysis.

Key words: Sweet potato, cluster analysis, genetic diversity, principal component analysis, Simple Sequence Repeats.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is a dicotyledonous plant that belongs to the family *Convolvulaceae* (Tortoe, 2010). In many developing countries it is an important food security crop (Korada et al., 2010). In Eastern Africa it is the third most important root crop grown after cassava and Irish potato (FAO 2011). Most sweet potato varieties

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grown in Africa are white, cream or yellow fleshed (Loebenstein and Thottappilly, 2009). Orange fleshed varieties were recently released in many countries and have become popular among the farmers and consumers. Sweet potato is used for human consumption, as a livestock feed, and in industrial processes to make alcohol, starch and other products such as noodles, candy, desserts, and flour (Lebot, 2010, Hazra et al., 2011). Sweet potato brings more income to farmers than any other root crop; both the roots, leaves and tender vines have economic and nutritional values (Antiaobong and Bassey, 2009). About 75% of African sweet potato production is concentrated in East Africa, especially around Lake Victoria where it is a basic subsistence crop (Kapinga et al., 1995; Gibson and Aritua, 2002). In Kenya, sweet potato production is practiced in the western, central and coastal areas of the country. Out of this, over 80% is grown in the Lake Victoria basin (Gruneberg et al., 2004) with Kakamega, Bungoma, Busia, Homa Bay, Rachuonyo and Kisii countries having high acreages of this crop.

Morphological identification (Huaman, 1992) has been widely used to characterise sweet potato accessions (Gichuru et al., 2006; Karuri et al., 2010). Morphological characterisation of parental genotypes for hybridisation schemes is not very efficient due to phenotypic plasticity and environmental effect on morphological traits (Price et al., 2003). According to Naylor et al. (2004), one can use molecular markers as tools to detect the extent and structure of genetic variation; provide insights into the diversity of crop varieties and potential contributions offered by their wild relatives; and to analyze the inheritance of key crop traits (including those that are subject to complex inheritance due to the involvement of numerous genes). Molecular markers concern the DNA molecule itself and, as such, are considered to be objective measures of variation. They are not subject to environmental influences; tests can be carried out at any time during plant development; and, best of all, have the potential of occurring in unlimited numbers, covering the entire genome (de Vicente and Fulton, 2003). Commonly used molecular markers include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter-Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR) (Williams et al., 1990). Such markers are phenotypically neutral, and not influenced by epistatic interactions (Koutita et al., 2005).

Use of microsatellites also called Simple Sequence Repeats (SSR) can be of great help in genetic diversity studies. SSRs are highly variable and evenly distributed throughout the genome (Hajeer et al., 2000). These are short, 2 to 8 nucleotide repeats such as CA or AGC, which are repeated in tandem up to hundreds of times at many independent loci, and are ubiquitous in eukaryote genomes (Lagarcrantz et al., 1993). These markers are easily automated, highly polymorphic, and have good analytical resolution, thus making them a preferred choice of markers (Matsuoka et al., 2002). These polymorphisms are identified by constructing PCR primers for the DNA flanking the microsatellite region (Hajeer et al., 2000; Godwin et al., 2001; Morgante et al., 2001). Since flanking DNA is more likely to be conserved, the microsatellite-derived primers can often be used with many varieties and even other species. Polymorphism is also based on the number of tandem repeat units (Godwin et al., 2001). These repeat motifs are flanked by conserved nucleotide sequences from which forward and reverse primers can be designed to PCR-amplify the DNA section containing the SSR (FAO/IAEA, 2002). SSRs can be exchanged easily between laboratories and multiplex reactions can be run to speed up the assay, where the products have non-overlapping size ranges. It is also possible to amplify SSRs using smaller amounts of DNA. There is a large collection of sweet potato germplasm available in Kenya. The diversity within it is largely unknown since only a few accessions have been characterized in previous studies (Gichuru et al., 2006, Karuri et al., 2010, Yada et al., 2010). If the germplasm is to be utilized in breeding programmes, or if potential duplicates within it has to be identified, then there is a need to undertake further characterization studies. The objective of this study was to characterize selected sweet potato accessions using microsatellite markers.

MATERIALS AND METHODS

Plant material used

A total of 68 sweet potato accessions randomly collected from various sources in East Africa (Kenya and Uganda) were used in this study (Table 1).

DNA extraction

DNA was extracted using a CTAB protocol modified from the Doyle and Doyle (1990) method. The modification involved omission of the ammonium acetate step and a longer DNA precipitation time of 12 h. The quality and quantity of the extracted DNA was checked by running it on a 1% agarose gel and using a nanodrop spectrophotometer. The DNA was then diluted to a working concentration of 30 ng/µl.

Microsatellite markers amplification

Polymerase Chain Reaction (PCR) amplification was done in an Applied Biosystem 2720 Thermo Cycler (Life technologies) using 13 microsatellite primer pairs (Table 2) obtained from Inqaba Biotechnical Industries Ltd. The amplification was performed in a 10µl reaction containing Gotaq Green Master Mix (Thermo scientific), 25 mM $MgCl_2$ (Promega), 10 µM of each primer (Inqaba Biotec), 25 ng DNA working concentration and ddH_20. The pre-amplification conditions were 45 cycles which included (i) initial denaturation at 94°C for 5 min, (ii) denaturation at 94°C for 30 s, (iii) annealing at 51°C for 30 s, (iv) extension at 72°C for 2 min,

Table 1. List of the 68 sweet potato accessions used in the study.

Accession number	Accession name[1]	Origin[2]	Flesh colour[3]
1	52 Nyakisumu	Landrace	Yellow-orange
2	56682-03	Kenya	Cream
3	Kenspot 1	Kenya	Yellow
4	Ejumula x New Kawogo 2	Uganda	Cream
5	Obugi	Landrace	Yellow-orange
6	Amina	Landrace	Orange
7	Ejumula	Uganda	Orange
8	Naspot x New Kawogo 3	Uganda	Yellow-orange
9	Mugande x New kawogo 3	Uganda	Cream
10	Mugande x new Kawogo 4	Uganda	Yellow-orange
11	36 Kalamb Nyerere	Landrace	Cream-yellow
12	Kunyikibuonjo	Landrace	Cream-white
13	Lungabure	Landrace	Cream-white
14	Polo yiengo	Landrace	Yellow
15	Sally boro	Landrace	Orange
16	1-Ujili	Landrace	Yellow
17	Mogesa Gikenja	Landrace	White
18	Naspot x New Kawogo 2	Uganda	Cream
19	Mbita	Landrace	Yellow
20	5-Nyandere	Landrace	Cream-Yellow
21	Odinga	Landrace	Yellow
22	Nangili	Landrace	Yellow-orange
23	Ejumula x New Kawogo 3	Uganda	Yellow
24	Nyarambe	Landrace	Cream
25	SPK 031	Kenya	Orange
26	9-Nduma	Landrace	Purple-cream
27	Nyamuguta	Landrace	Cream-white
28	Wera	Landrace	Yellow
29	Oduogo jodongo	Landrace	White
30	K/KA/2002/12	Kenya	White
31	K/KA/2004/215	Kenya	Yellow
32	Fumbara jikoni	Landrace	Cream
33	K-117	Kenya	White
34	292-H-12	Kenya	Yellow-cream
35	Mwavuli	Landrace	Cream
36	Mugande	Rwanda	White
37	SPK 004	Kenya	Orange
38	29 Kuny kibuonjo	Kenya	Yellow
39	Santo Amaro	Brazil	Cream
40	62 Odhiogo	Landrace	Yellow
41	Naspot x New Kawogo 1	Uganda	Cream
42	Ejumula x New Kawogo 4	Uganda	Yellow-orange
43	Karunde	Landrace	Cream
44	Kibuonjo	Landrace	Cream-white
45	12 Maooko	Landrace	Cream
46	Sinia	Landrace	Yellow
47	Kenspot 2	Kenya	White
48	Kemb 10	Kenya	Yellow
49	Ejumula x New kawogo 1	Uganda	Cream

[1*]All the crosses in this paper are F1 hybrids from a polycross obtained from National Crops Resources Research Institute (NaCCRI), Uganda.[2*]Any accession whose origin we could not determine was assigned as a landrace.

Table 1. Contd.

Accession number	Accession name[1]	Origin[2]	Flesh colour[3]
50	Kenspot 5	Kenya	Orange
51	55 Nganyomba	Landrace	Cream
52	Kenspot 3	Kenya	Orange
53	Nyakagwa	Kenya	Cream
54	24 Kampala	Uganda	Yellow-orange
55	91/2187	Kenya	Yellow
56	Nyawo nyathi odieyo	Landrace	Orange
57	Vita	Landrace	Cream
58	Gachaka	Landrace	Yellow-orange
59	Kenspot 4	Kenya	Orange
60	Naspot 1	Uganda	Yellow
61	Mugande x New kawogo 1	Uganda	Yellow
62	SPK 013	Kenya	White
63	Nyautenge	Landrace	Cream
64	Tainung	Taiwan	Orange
65	Mugande x New kawogo 2	Uganda	Cream
66	Bungoma	Uganda	Cream
67	Alupe-OR	Landrace	Orange
68	Fundukhusia	Landrace	Yellow-orange

and (v) final extension 72°C for 10 min. After amplification, 10ul of each of the amplicons was loaded on a 2% agarose gel (Bioline). Gel electrophoresis was done at a voltage of 80V and a current of 400mA for 1 hour in Tris Borate EDTA buffer. The amplicons were visualised as fluorescent bands under UV light on an Ebox VX5 Transilluminator (Wilber Lourmat). The size of the amplified markers was determined by using O'gene ruler green ready to use 100bp or 1Kb molecular ladder (Thermo Scientific). For each sample, the presence of a band (allele) was recorded as either present or absent.

Statistical analysis

PCR bands (alleles) were scored for all the markers. The data was entered on an excel sheet in a binary form with '0' indicating absence of an allele while '1' its presence. However, for analysis on Popgene the scoring was '2' for presence of an allele and '1' for absence. Any extra amplification on any marker was scored as a separate allele. The data was then analysed using DARwin version 6 software (Perrier and Jacquemoud-Collet, 2006) for Unweighted Pair Group Method with Arithmetic means (UPGMA) tree and Principal Component Analysis (PCoA) while Powermarker version 3 software (Liu and Muse, 2005) was used to compute markers summary statistics. The number of effective alleles was computed using Popgene software (Yeh et al., 1997).

RESULTS

Major allele frequency

The major allele frequency value ranged from 0.5882 to 0.9412 with a mean of 0.7563. Marker JB1809a had the lowest major allele frequency while marker J67b and J67c had the highest major allele frequency (Table 3). These values were quite high with all the values above 0.5. The total number of alleles amplified was 21.

Gene diversity

The gene diversity values ranged from 0.1107 to 0.4844 with a mean value of 0.3384. Markers J67b and J67c had the lowest values while marker JB1809a had the highest value (Table 3).

Polymorphic information content

The PIC values ranged from 0.1046 to 0.3671 with a mean value of 0.2723. Markers J67b and J67c had the lowest values while marker J1809a had the highest value (Table 3).

Effective number of alleles

The number of effective alleles values ranged from 1.0921 to 1.9396 with a mean value of 1.5513. Markers J67b and J67c had the lowest values while marker J1809a had the highest value.

Phylogenetic tree

A UPGMA tree was constructed based on dissimilarity matrix computed using Jaccards coefficient. The

Table 2. List of microsatellite markers and primer pairs used in the study.

Primer	Sequence	Repeat Motif	At (°C)	Reference
IBR03	F GTAGAGTTGAAGAGCGAGCA R CCATAGACCCATTGATGAAG	(GCG)5	53	Benavides (unp.)
IBR12	F GATCGAGGAGAAGCTCCACA R GCCGGCAAATTAAGTCCATC	(CAG)5A	55	Benavides (unp.)
IB242	F GCGGAACGGACGAGAAAA R ATGGCAGAGTGAAAATGGAACA	(CT)3CA(CT)11	54	Buteler et al., 1999
IB275	F GAGTTCCAAAGAGAAGAGTGGAG R AAGCCTACCCGAGAGATAACC	(CT)27	56	Buteler et al., 1999
J175	F ATCTATGAAATCCATCACTCTCG R ACTCAATTGTAAGCCAACCCTC	(AATC)4	54	Solis et al. (unp.)
IB316	F CAAACGCACAACGCTGTC R CGCGTCCCGCTTATTTAAC	(CT)3C(CT)8	55	Buteler et al., 1999
IB324	F TTTGGCATGGGCCTGTATT R GTTCTTCTGCACTGCCTGATTC	*	53	Tseng et al., 2002
IBCIP	F CCCACCCTTCATTCCATTACT R GAACAACAACAAAAGGTAGAGCAG	(ACC)7A	56	Yanez, 2002
IBJ522	F ACCCGCATAGACACTCACCT R TGACCGAAGTGTATCTAGTGG	(CAC)6-7	56	Solis et al. (unp.)
IBS07	F GCTTGCTTGTGGTTCGAT R CAAGTGAAGTGATGGCGTTT	(TGTC)7	53	Benavides (unp.)
J67	F CACCCATTTGATCATCTCAACC R GGCTCTGAGCTTCCATTGTTAG	(GAA)5	56	Solis et al. (unp.)
JB1809	F CTTCTCTTGCTCGCCTGTTC R GATAGTCGGAGGCATCTCCA	(CCT)6(CCG)6	57	Solis et al. (unp.)
IB297	F GCAATTTCACACACAAACACG R CCCTTCTTCCACCACTTTCA	(CT)13	54	Buteler et al., 1999

*At: Annealing temperature.

dissimilarity matrix was computed using 1000 bootstraps. The tree revealed four major clusters (Figure 1). The four clusters had 19, 4, 31 and 14 accessions for clusters I, II, III and IV, respectively.

Principle component analysis

Principal component analysis (PCoA) showed that 68 accessions fell into four major clusters. The first three clusters had 7, 50 and 10 accessions, respectively, while accession 64 formed a cluster of its own (Figure 2).

DISCUSSION

Marker assisted breeding (MAB) is increasingly becoming a crucial part of modern plant breeding in Africa. Genetic diversity using various marker platforms, but more commonly microsatellite markers, is slowly becoming a

Table 3. Table of summary statistics of the 21 alleles amplified in the sweet potato accessions.

Marker	Major allele frequency	Sample size	Allele no.	Availability	ne*	Gene diversity	PIC
IBR03	0.6176	68.0000	2.0000	1.0000	1.8951	0.4723	0.3608
IBR12	0.7794	68.0000	2.0000	1.0000	1.5241	0.3439	0.2847
IB242	0.6471	68.0000	2.0000	1.0000	1.8408	0.4567	0.3524
IB275	0.6765	68.0000	2.0000	1.0000	1.7785	0.4377	0.3419
J175	0.6765	68.0000	2.0000	1.0000	1.7785	0.4377	0.3419
J175b	0.8971	68.0000	2.0000	1.0000	1.1918	0.1847	0.1676
IB297	0.7794	68.0000	2.0000	1.0000	1.4859	0.3439	0.2847
IB316	0.7647	68.0000	2.0000	1.0000	1.5241	0.3599	0.2951
IB324	0.7059	68.0000	2.0000	1.0000	1.7101	0.4152	0.3290
IBCIP	0.6029	68.0000	2.0000	1.0000	1.9187	0.4788	0.3642
IBCIPb	0.8382	68.0000	2.0000	1.0000	1.3349	0.2712	0.2344
IBCIPc	0.7794	68.0000	2.0000	1.0000	1.4859	0.3439	0.2847
IBJ522	0.6029	68.0000	2.0000	1.0000	1.9187	0.4788	0.3642
IBJ522b	0.8971	68.0000	2.0000	1.0000	1.1918	0.1847	0.1676
IBS07	0.7059	68.0000	2.0000	1.0000	1.6741	0.4152	0.3290
J67a	0.6029	68.0000	2.0000	1.0000	1.9187	0.4788	0.3642
J67b	0.9412	68.0000	2.0000	1.0000	1.0921	0.1107	0.1046
J67c	0.9412	68.0000	2.0000	1.0000	1.0921	0.1107	0.1046
JB1809a	0.5882	68.0000	2.0000	1.0000	1.9396	0.4844	0.3671
JB1809b	0.9265	68.0000	2.0000	1.0000	1.1245	0.1362	0.1270
JB1809c	0.9118	68.0000	2.0000	1.0000	1.1577	0.1609	0.1480
Mean	0.7563	68.0000	2.0000	1.0000	1.5513	0.3384	0.2723

ne* = Effective number of alleles.

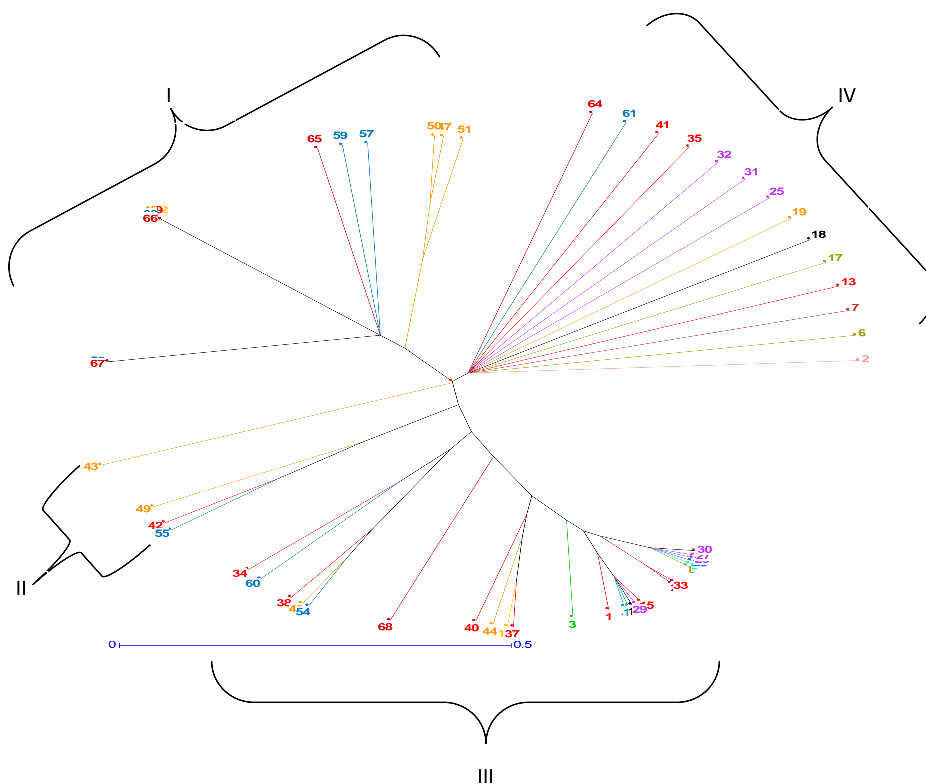

Figure 1. UPGMA tree based on Jaccard's coefficient of dissimilarity.

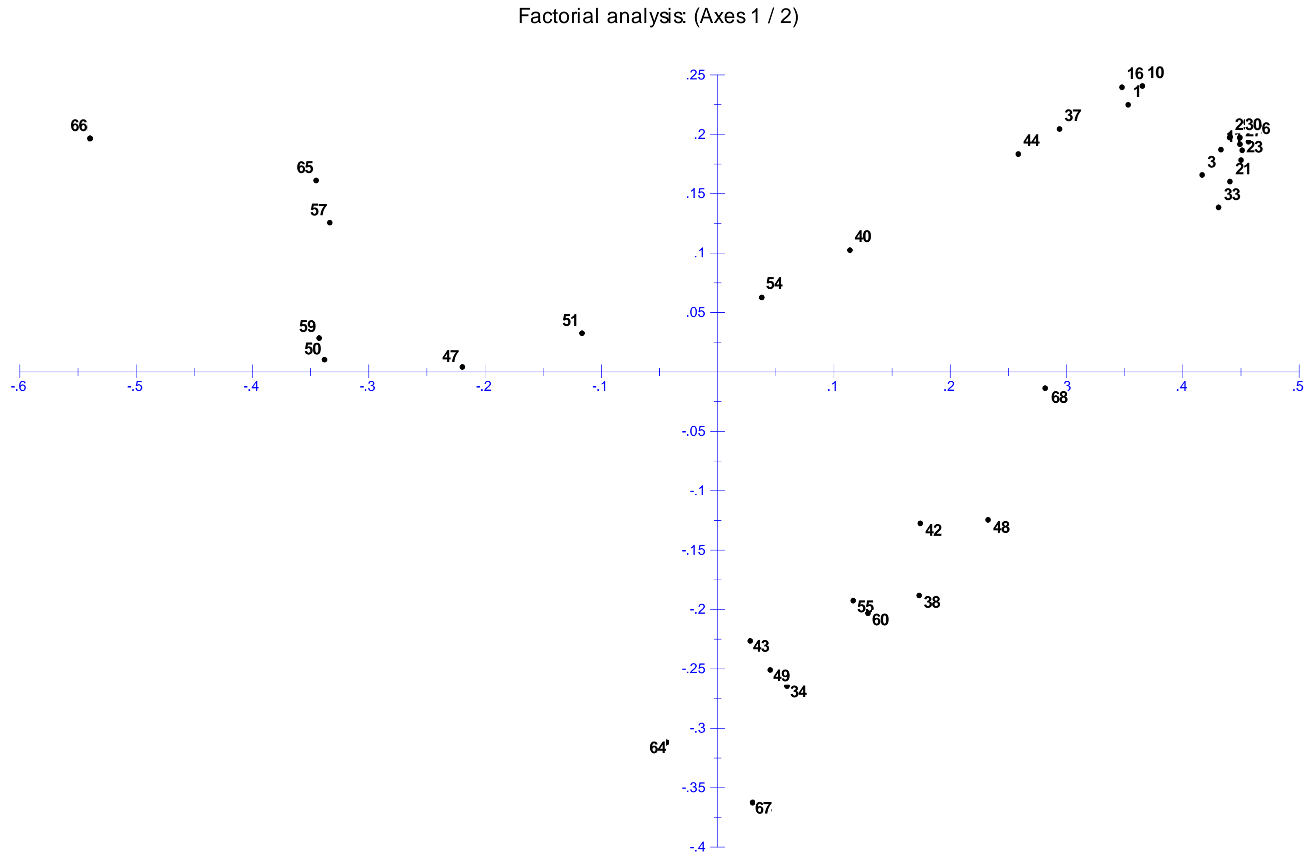

Figure 2. A 1 by 2 factorial analysis (PCoA) diagram of the 68 sweet potato accessions.

common molecular biology tool applied on food crops breeding. The application of MAB increases the efficiency of breeding programs hence reduce the time required to release superior food crop varieties. However, the adoption rate of MAB in breeding programs in most African countries is still very slow due to financial and technical constraints. Genetic diversity studies using molecular phylogenetics form one core application of MAB, most especially on major food crops. Such studies are very important in selecting parents for hybridisation or crossing experiments aimed at improving the food crops varieties. Microsatellite-based genetic diversity studies on East African sweet potato accessions have been done before (Gichuru et al., 2006; Yada et al., 2010; Karuri et al., 2010). In this study, 68 sweet potato accessions were assessed for genetic diversity using 12 primer pairs which amplified a total of 21 alleles.

The UPGMA tree produced four major clusters with cluster three having most accessions overlapping. The tree could only give the general germplasm relatedness and diversity. However, the PCoA gave better resolution in terms of revealing the germplasm relatedness and diversity. From the PCoA the accessions clustered into 4 main axis with accession 64 falling on its own axis. This accession may have clustered alone due to its origin (Taiwan). The PCoA further showed the clustering of the accessions with several accessions overlapping indicating possible duplicates. This is important as identification of duplicates and genetically distinct accessions can help in selecting parents for hybridisation experiments. It is therefore advisable to use both the phylogenetic tree and the PCoA in studying the genetic diversity of germplasm since they complement each other.

The PIC values obtained in this study were quite low since all the values were below 0.5. This might have a direct implication on the discriminatory power of the markers on the accessions used in this study. However, the PIC value of 0.329 for marker IBS07 was higher than the 0.23 obtained by Yada et al. (2010) but lower than the 0.33 obtained by Karuri et al. (2010). The gene diversity values followed the same pattern implying low marker polymorphism. This could be due to the low genetic diversity of sweet potato considering the fact that it is a clonally propagated crop. Another explanation is that farmers in different regions tend to give a particular variety different local name, hence when a breeder collects accessions for hybridization or genetic diversity studies, they might collect the same variety under different names. This means that the breeder might use the same variety as the male as well as the female parent in hybridization leading to inbreeding and low genetic

diversity. It is therefore important to do germplasm characterization before making crosses to determine the genetic diversity of the parental genotypes.

Conclusion

From the results above, SSR markers were successfully used to effectively characterise the selected sweet potato germplasm. The study also revealed that the markers can effectively discriminate the different accessions as seen from the phylogenetic trees and the factorial analysis. Cluster analysis also indicated possible duplicates with several accessions overlapping. It is therefore important to incorporate SSR marker analysis in the selection of genetically distinct germplasm and to identify duplicates in sweet potato germplasm conservation.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Abbreviations: MAS, Marker Assisted Selection; **RFLP,** restriction fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphism; **ISSR,** inter-simple sequence repeats; **SSR,** simple sequence repeats; **PCR,** polymerase chain reaction; **UPGMA,** Unweighted pair group method with Arithmetic means; **PCoA,** principal component analysis.

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Full Length Research Paper

Impact of the application of humic acid and sodium nitroprusside on nickel toxicity: Analysis of relative gene expression

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Nickel (Ni) is an essential micronutrient for plants but in high concentrations may turn toxic. This paper discusses the potential role of humic acid (HA) and sodium nitroprusside in modulating or preventing oxidative stress in rice plants. Three genes [superoxide dismutase (SOD) glutathione reductase (GR) and ascorbate peroxidase (APx) were selected for an expression study using a real time PCR technique. Three different treatments (T1 = nickel [nickel chloride ($NiCl_2 \cdot 6H_2O$)] 300 mg L^{-1}, T2 = nickel-humic acid, T3 = nickel-sodium nitroprusside) were used to determine the effect of humic acid and sodium nitroprusside on nickel toxicity in rice plants. Rice plants grown in T2 appeared green and well developed. In leaves and roots, the expression of superoxide dismutase and ascorbate peroxidase was higher in T3 (nickel-sodium nitroprusside); glutathione reductase expression in roots was lower in T1 (sand with Ni solution) compared to T2 (nickel 300 mg L^{-1} and HA) where the expression was higher; significant differences were found between both treatments. In leaves, the behavior of this gene was similar in all treatments. This research suggests that nickel toxicity cannot be diminished when HA or SNP are used, and they induce oxidative stress in rice plants.

Key words: Nickel toxicity, heavy metals, gene expression, oxidative stress.

INTRODUCTION

Soil contamination with heavy metals like lead (Pb), Cadmium (Cd) and nickel (Ni) is an environmental problem worldwide because these metals may bio-accumulate and they do not have specific metabolic functions for living beings. This pollution is mainly due to the intense industrialization and urbanization (Wei and Yang, 2010; Yaylali-Abanuz, 2011; Mireles et al., 2012). Nickel is a ubiquitous trace metal and compounds such as nickel acetate, nickel carbonate, nickel hydroxide and nickel oxide are used in a wide range of industrial processes.

These compounds ultimately accumulate in soil and environment, and can be easily absorbed by plants. Thus, they can enter the food chain and cause deleterious effects to animals and humans (Cempel and Nikel, 2006). Human exposure to nickel and its compounds has the potential to produce a variety of pathological effects, which may include cutaneous inflammations such as swelling, reddening, eczema and itching on skins, and may also induce allergy reactions and teratogenicity in the human body. The most concerning adverse health effects due to nickel exposure are lung fibrosis and lung cancer (Zhao et al., 2009). Higher plants have developed a series of protective mechanisms to counteract Ni-toxicity and to control the generation of excessive reactive oxygen species (ROS). These mechanisms include anti-oxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Xu et al., 2010).

In rice plants, an increase in Ni levels produces diverse toxicity symptoms such as chlorosis and necrosis (Samantaray et al., 1997); also, the number of lateral roots considerably decreases (Seregin and Kozhevnikova, 2006). With the development of the global economy, both type and content of heavy metals in the soil caused by human activities have gradually increased in recent years, which have resulted in serious environmental deterioration (Su et al., 2014). One way to diminish heavy metals toxicity on plants is the use of natural products, such as humic acid (HA) and nitric oxide donors like sodium nitroprusside (SNP). Application of humic acid not only effectively improves soil physical and chemical properties, to provide a more suitable environment for plant growth, but also significantly reduces the use of chemical fertilizers and pesticides in soils. As an important way for increasing yield in agricultural production, the use of humic acid can also accelerate remediation of contaminated soil by heavy metals (Xu et al., 2010).

In the case of the SNP the number of studies that have examined the exogenous NO effects on reducing heavy metal toxicity in plants has increased. Application of SNP under different heavy metals toxic conditions may protect rice seedlings from Cd and As (arsenic) (Panda et al., 2011; Singh et al., 2009). These studies strongly suggest that exogenous NO can protect plants from the harmful impacts of toxic heavy metals concentrations. The aim of this research was to evaluate the effect of Humic Acid and Sodium Nitroprusside on diminishment of toxicity in rice plants exposed to nickel through a gene expression analysis of some gene related to antioxidant activity in plants.

Table 1. Composition of the Hoagland`s nutrient solution.

Component	Stock Solution g L^{-1}
Macronutrients	
2 M KNO_3	202
1 M $Ca(NO_3)_2•4H_2O$	236 g $0.5L^{-1}$
Iron (Sprint 138 iron chelate)	15
2 M $MgSO_4•7H_2O$	493
1 M NH_4NO_3	80
Micronutrients	
H_3BO_3	2.86
$MnCl_2•4H_2O$	1.81
$ZnSO_4•7H_2O$	0.22
$CuSO_4•5H_2O$	0.051
$H_3MoO_4•H_2O$ or	0.09
$Na_2MoO_4•2H_2O$	0.12
Phosphate	
1 M KH_2PO_4 (pH to 6.0)	136

MATERIALS AND METHODS

Plant material

Rice (*Oryza sativa*) var. Tetep was kindly provided by National Institute of Agricultural Science (INCA, Cuba). Seeds were sterilized with 5% sodium hypochlorite for 15 min, and then rinsed with distilled water for three times. Seeds were sown in pots filled with distilled water and covered with a thin cloth to avoid water evaporation. Pots were placed in a phytotron with a temperature of 35°C and a relative humidity of 32 to 35%. After one week seedlings were transplanted to 1 L pots filled with sand and Hoagland's nutrient solution (600 mL) (Table 1) (four seedlings by pots) with Ni [nickel chloride ($NiCl_2·6H_2O$)] 300 mg L^{-1} (T1 = control), ($NiCl_2·6H_2O$) 300 mg L^{-1} + HA (T2), or ($NiCl_2·6H_2O$) 300 mg L^{-1} + SNP (T3). HA were used at 46 mg L^{-1} (Garcia et al., 2012) and SNP at 7.2 mg L^{-1} (Zhao et al., 2013). The experiments were carried out in a glasshouse under natural daylight (September to December 2014) with temperatures in the range of 20 to 30°C. The Hoagland solution with Ni was changed weekly, and the total volume was completed with water once a week. Roots and leaves were collected after 30 days and stored at -80°C for further analysis.

Total RNA isolation

Total RNA was extracted according to Gao et al. (2001) method

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Abbreviations: HA, Humic acid; **SOD,** superoxide dismutase; **GR,** glutathione reductase; **APx,** ascorbate peroxidase; **ROS,** reactive oxygen species; **CAT,** catalase; **SNP,** sodium nitroprusside **PCR,** polymerase chain reaction.

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Table 2. Sequences of the primers used for real-time PCR.

Oligo name	Length (pb)	Tm (°C)	Sequence (5`-3`)
APx			
OsAPx1 mRNA F	20	64.8	5'GCGACTACAAAGGGAGGGTC3'
OsAPx2 mRNA R	20	64.3	5'TTAGATCCAGGCTCCTGTGC3'
GR			
OsGr2 mRNA F	24	64.3	5'GGTGATGAACCTACCAAACCAGAT 3'
OsGr3 mRNA R	19	66.9	5' GGGTGGTTGGGAGAAAACG 3'
SOD			
OsSOD3 mRNA F	20	66.8	5' CTCCAGAGCGCCATCAAGTT 3'
OsSOD5 mRNA R	23	63.3	5'TCCAGAAGATCGAATGATTGACA 3'

($LiCl_2$) using an NTES buffer (0.2 M Tris-HCl pH 8.0; 25 mM EDTA, 0.3 M NaCl; 2 % SDS). Leaves and roots samples were ground in N_2 and homogenized in a mixture containing 4.5 mL NTES buffer and 3 mL of phenol:chloroform (1:1). Homogenized samples were centrifuged at 12,000 × *g* for 10 min at 4°C and supernatant was transferred to a new tube. Total RNA was precipitated by adding 1/10 volume of 2 M sodium acetate pH 4.8 ($NaOAc_{DEPC}$) and one volume of cold isopropanol. Samples were placed at -20°C for 2 h and then centrifuged at 12,000 × *g* for 10 min. Pellets were re-suspended in 2.5 mL of H_2O-DEPC and precipitated again by the addition of 2.5 mL of 4 M lithium chloride pH 4.8 ($LiCl_2$-DEPC). After centrifugation at 12,000 × *g* for 10 min, pellets were washed with 70% ethanol and dissolved in 0.1 mL H_2O-DEPC. RNA was then stored at -80°C until use. The RNA concentration was determined using a NanoDrop Spectrophotometer (NanoDrop 1000 Thermo Fisher Scientific) prior a complementary DNA (cDNA) experiment.

cDNA first strand synthesis

The first strand of cDNA was synthesized using a Taq Man Reverse Transcription kit (Applied Biosystems, Inc.) A polymerase chain reaction (PCR) of three sequential steps of one cycle was performed at 42°C for 5 min, 50°C for 50 min and another at 70°C for 15 min.

Analysis of the expression levels by real time-PCR

The differential expression of some genes involved in oxidative stress response [Ascorbate peroxidase (OsAPx1, OsAPx2), glutathione reductase (OsGR2, OsGR3) and superoxide dismutase (OsSOD3, OsSOD5) was confirmed by real-time PCR. The primers were designed using Primer Express 2.00 (Applied Biosystems software), based on sequences retrieved from the National Center of Biotechnology Information (NCBI) database. Primers sequences specificity and melting curve of the final PCR reaction were analyzed through TIGR (http://rice.plantbiology.msu.edu) and with NCBI (http://www.ncbi.nlm.nih.gov). All primers used are listed in Table 2. RT-PCR was performed using a Platinum®SYBR® Green qPCR Super MIX-UDG (Invitrogen) in a reaction mixture of 20 µL containing: 0.5 µL of each primer (10 $pmol \cdot L^{-1}$) (Table 2), 10 µL of SYBR Green qPCR Super MIX-UDG, 2 µL of cDNA and 7 µL of RNase-free water, with 48-wells plates and the standard cycling program. PCR reactions were as follows: 10 min at 95°C, 40 amplification cycles at 95°C for 15 s and 60°C for 1 min (annealing, extension and fluorescence detection), followed by the "melting curve" accomplished by the increase in temperature at intervals of 0.3°C, from 60 to 95°C in order to verify the specificity of the reaction. Reaction conditions (10 mL volumes) were optimized to increase PCR efficiency by changing the primer concentration and annealing temperature to minimize primer-dimer formation. The absence of primer-dimers or accumulation of non-specific products was checked by melting-curve analysis. PCR efficiency was determined through a standard curve with serial dilutions of cDNA using Actin primer. The housekeeping gene Actin was used as an internal reference for normalization of gene as the "driver". Each sample was analyzed in triplicated. The rate of gene expression was calculated using the delta-delta CT (ΔΔCT) method (Livak and Schmittgen, 2001). At first, the threshold cycles (CT) of the duplicate PCR results of each gene were averaged and used for quantification of the transcripts. Then, the average of the CT value of the Ubiquitin (*UBQ5*) gene was subtracted from the average of the CT value of the target gene to obtain the ΔCT value. The 2ΔΔCT value was given to estimate the relative expression rate of each gene. Each value was obtained from two independent experiments. A standard deviation was given to each value and the results were analyzed by the Student's t-test. A P-value of ≤0.05 was considered significant.

RESULTS

Phenotypical differences between treatments

Reductions in plants growth and chlorosis of leaves was observed in T1 (Figure 1A) while in T2 (Ni 300 mg L^{-1}+ HA) phenotypical characteristics of rice seedling like plant growth and area of leaves were better (Figure 1B) compared to T1 and T3 (Figure 1C). In T2 (nickel 300 mg L^{-1}+ HA) plants grew well, leaves showed normal size and green color, maybe because HA improves plant development in environments polluted with heavy metals and also reduces availability and mobility of heavy metals in the soils.

Analysis of the expression levels of ascorbate peroxidase (OsAPx1, OsAPx2), glutathione reductase (OsGR2, OsGR3) and superoxide dismutase (OsSOD3, OsSOD5) by real time PCR

Real Time PCR analysis showed different expression

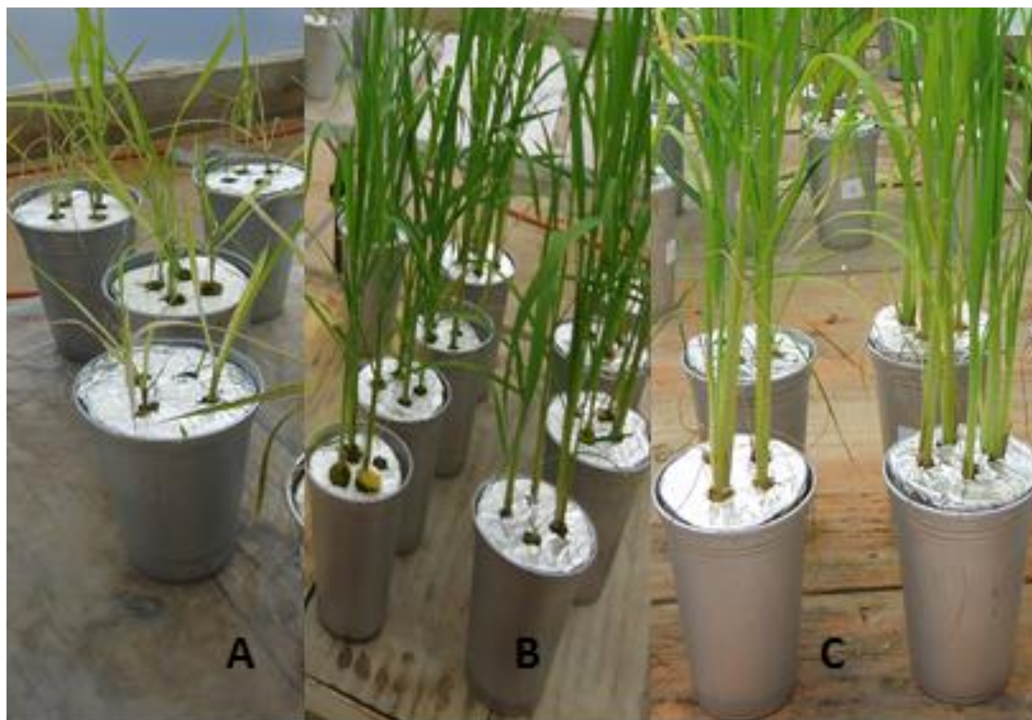

Figure 1. Phenotypical characteristics of rice (*O. sativa*) seedlings. **A,** Hoagland's nutrient solution + Ni 300 mg·L^{-1} (T1). **B,** Ni 300 mg·L^{-1} + HA (T2). **C.** Ni 300 mg·L^{-1} + SNP (T3).

profiles of APx, GR and SOD in roots and leaves of rice variety Tetep (Figure 2A, B, C). According to Figure 2A, there was a significant expression of ascorbate peroxidase (APx) in roots in T3 (nickel 300 mg L^{-1}+ SNP), indicating the possible toxicity of SNP, while in T1 (control only with Ni) and T2 (nickel 300 mg L^{-1}+ HA) expression in this organs was lower. Significant differences in leaves for this variable were not found. The expression of GR is shown in Figure 2B. Significant differences were found, being the expression of this gene in T2 higher in roots compared to T1 and T3. In leaves, the expression of this gene was similar in all treatments. The induction of SOD was similar in root and leaves being significantly higher in T3 (nickel 300 mg L^{-1} + SNP) and lower in T2 (nickel 300 mg L^{-1} + HA) in both organs analyzed.

DISCUSSION

In this research, the effect of HA and SNP in rice plants with Ni heavy metal was investigated according to the expression of some important gene related with oxidative stress in plants. Several studies showed that the toxic effect of Ni causes various physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in a variety of plant species (Zornoza et al., 1999; Pandey and Sharma, 2002), and specifically in rice (Samantaray et al., 1997). The toxic effects of Ni and some other heavy metals are manifested first by the inhibition of plant growth (Seregin et al., 2003; Nagajyoti et al., 2010) as was shown in this research where rice plants showed growth affectation with Ni at 300 mg·L^{-1} (Figure 1A). Ni has strong influence on metabolic reactions in plants, and it can induce reactive oxygen species (ROS) that can lead to oxidative stress (Sreekanth et al., 2013). The application of humic substances to plants has been proved to stimulate their biochemical-physiological mechanisms, growth and development. Humified materials exhibit structural characteristics that allow interactions with heavy metal cations dissolved in aqueous environments (Garcia et al., 2012). APx and SOD expression was lower in root when HA was used, possibly because HA is considered as organic matter and contributes to change a particular form of toxic elements, in this case Ni.

SNP is a chemical compound used as Nitric oxide (NO) donor (Beligni and Lamattina, 2002). Several studies have shown the protective effect of NO against abiotic stress and also its mediated reduction of ROS in plants (Hsu and Kao, 2004). However, in this study with Ni as a heavy metal, the oxidative stress increased with this substance significantly in roots of rice plants, as evidence of the high expression of APx and SOD. APx gene expression in the leaves of rice plants was higher than in roots in all treatments. In roots, significant values were found in T3 compared to the other two treatments where exogenous SNP significantly induced activities of APX.

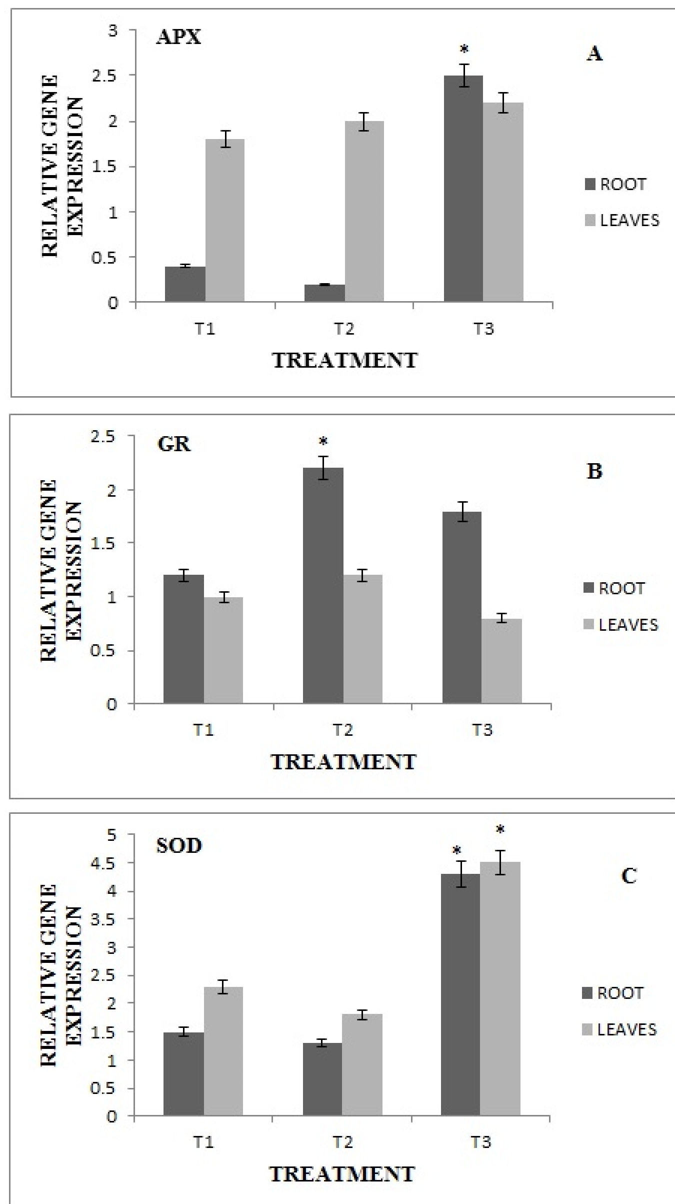

Figure 2. Relative expression of transcriptionally-ascorbate peroxidase (APx). **(A)** glutathione reductase (GR). **(B)** Superoxide dismutase (SOD). **(C)** Rice seedlings grown in different treatments in root and leaves. Each value is the mean ± standard error of three replicates. Data points marked with asterisk (*P ≤ 0.05) indicate statistically significant differences.

This discrepancy in expression for the OsAPx genes might be due to differences in organs (Hong et al., 2007).

In leaves, APx activity increased in all treatments because cytosolic APx (OsAPx1, OsAPx2) is essential for oxidative protection of chloroplasts against stress (Miller et al., 2007; Koussevitzky et al., 2008; Maruta et al., 2010). Also, the H_2O_2 quantity significantly increased in wheat leaves under Ni-stress (Gajewska and Skłodowska, 2007); this behavior can be extrapolated to rice because both plants belong to the same family (grasses). Ascorbate peroxidase (APX) activities increased in leaves under Ni-stress because this enzyme may play a significant role in the cleaning of H_2O_2 from the leaves of Ni-stressed plants (Gajewska and Skłodowska, 2007); this can explain why the expression of this gene is higher in leaves even with HA and SNP.

According to Shigeoka et al. (2002), APX activity generally increases in response to environmental stress as occurred in this study at a molecular level. Normally, APx2 in *Arabidopsis thaliana* L. is inducible mainly under extreme light or heat stress conditions (Karpinski et al., 1999; Panchuk et al., 2002) but in this research OsAPx gene is inducible by Ni heavy metal. The cDNA of OsGR2 was first isolated in 1998. Subcellular fractionation showed that the OsGR2 protein is localized primarily in cytosol; mRNA and protein of OsGR2 were observed mainly in roots and calli but little in leaf tissues (Kaminaka et al., 1998); that is why the activity is higher in this tissue (roots). Together with OsGS1, OsGR3 is a specific *Poaceae Isoform* targeted to chloroplasts and mitochondria (Tsung-Meng et al., 2013). One GR cytosolic isoform (OsGR2) and two chloroplastic isoforms (OsGR1 and OsGR3) have been identified in rice (Rouhier et al., 2006; Bashir et al., 2007). The lower expression of GR in T1 (sand with Ni solution) may be caused by the possible temporal expression of this gene, mainly transitional. However, this response also could be a consequence of GR-related transcriptional activity gene (Perez et al., 2013).

Recently, it was reported that HA applied to the roots of rice plants stimulated several enzymatic mechanisms associated with the antioxidative defense system (Garcia et al., 2012), as shown in Figure 2B where this substance increases GR expression in rice roots, but this increase indicates oxidative stress. Tsung-Meng et al. (2013) suggested that, through an expression analysis the involvement of OsGR3 is in response to salt stress and salicylic acid (SA), a signal molecule of systemic resistance. They also showed that OsGR3 is a functional GR. From studies using transgenic plants, it has been proved that GR plays a prominent role in conferring resistance to oxidative stress caused by drought, ozone, heavy metals, high light, salinity, cold stress, etc. There has been found an enhanced GR activity in *A. thaliana*, *Vigna mungo* L., *Triticum aestivum* L., *Capsicum annuum* L. and *Brassica juncea* L. after cadmium treatments. Sharma and Dubey (2005) have found an increased GR activity in *O. sativa* seedlings during drought conditions. All these results show the role of this enzyme in plant protection against abiotic stress. The significant increase in GR gene expression found in rice roots with Humic Acid (T2) means an increase in oxidative stress. In this case HA and SNP enhance the expression of this gene mainly in roots, meaning that the aim to diminish Ni toxicity with these products was not accomplished.

In this research, the involvement of OsGR2 and OsGR3

in relation to Ni stress is suggestive. Further functional studies are required to clarify whether OsGR2 and OsGR3 are involved in heavy metal-stress tolerance. The activation of SOD could be useful to reduce O_2^- accumulation, decrease H_2O_2 and alleviate some heavy metals stress (Wang et al., 2008). The expression of SOD (Figure 2C) increases significantly in T3 for both organs (roots and leaves) revealing the presence of oxidative stress. Some researches obtained positive results with the use of SNP, like Yu et al. (2013) who found that in cucumber plants, exogenous application of SNP increases the antioxidant capacity in this crop. Also, Zhao et al. (2013) in rice under cadmium (Cd) toxic conditions demonstrated that applications of SNP may protect rice seedlings from Cd stress (Zhao et al., 2013). The concentration of SNP used in this research, maybe the interaction with Ni, induced increases in gene expression related with antioxidant enzymes. Bai et al. (2015) used SNP in rice seedlings at different concentrations in the presence of lead (Pb) and they found that Pb- induced oxidative damage was reduced with 50, 100 and 200 μM of SNP however, 400 μM of SNP had no obvious alleviating effect in Pb toxicity. These authors demonstrated the effect of SNP concentration on heavy metal toxicity. In this specific case, Ni effects may not be mediated only by oxidative stress, but by some additional mechanisms susceptible to NO.

Conclusion

Exogenous HA and SNP application had no effect on diminishment of Ni toxicity in rice var. Tetep at molecular level. Based on the results, it can be concluded that the effects of SNP did not alleviate Ni stress in rice, may be due to NO from it, and the mechanism and interaction of $NiCl_2$ and SNP should be further investigated.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Screening of successive extracts of *Amorphophallus konjac* for antibacterial activity

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***Amorphophallus* is an Aroid family member, native to Asia. *Amorphophallus konjac* K. Koch ex N.E.Br. is also known as snake plant due to snake like outlines on its stem. In Mount Abu the plant is grown in wild and known for its toxic principles. In Traditional Chinese System of Medicine (TCM), it was mentioned that gel prepared from flour has been used in detoxification, tumor suppression, phlegm liquefaction and skin disorders. In the present research work, attempts were made to authenticate and validate the ethno-medicinal potentials of *A. konjac* antimicrobial activity which could be alternate for current synthetic antimicrobial agents. The plant was selected for screening of antimicrobial efficacy against eight selected bacterial strains viz. *Staphylococcus aureus* (ATCC- 2921), *Klebsiella pneumoniae* (ATCC 700603), *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Proteus mirabilis* (ATCC 12453), *Enterobacter cloacae* (ATCC-13047), *Enterococcus faecalis* (ATCC 29212) and *Streptococcus pneumoniae* (ATCC 6305). The results of antimicrobial activity of crude dicholormethane (DCM), ethyl acetate, chloroform and methanol were significant. DCM extract C (10 mg/disc) possess maximum efficacy against *S. pneumoniae* (IZ = 20 mm; AI = 1.25). The main cause of community acquired pneumonia and septicemia in HIV infected patients is caused by *S. pneumoniae* microorganism. Further, bioactivity guided fractionation of pure compounds from DCM extract of *A. konjac* can lead to work as novel antibiotic in future. Therefore, the extract can also be used for isolation of volatiles compounds with potentials so that the extract / active fraction / pure compounds can be used as nasal spray in future therapeutics.**

Key words: *Amorphophallus konjac*, antibacterial activity, antimicrobial agents, ethnomedicinal plant.

INTRODUCTION

Amorphophallus konjac possess vast history of consumption in tropical and subtropical Asia as a potentials source of food and also included in Traditional Chinese Medicine (TCM) (Liu et al., 1998).

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Abbreviations: ATCC, American type culture collection; **TCM,** traditional Chinese system of medicine; **DCM,** dicholormethane; **NAM,** nutrient agar medium; **AI,** activity index.

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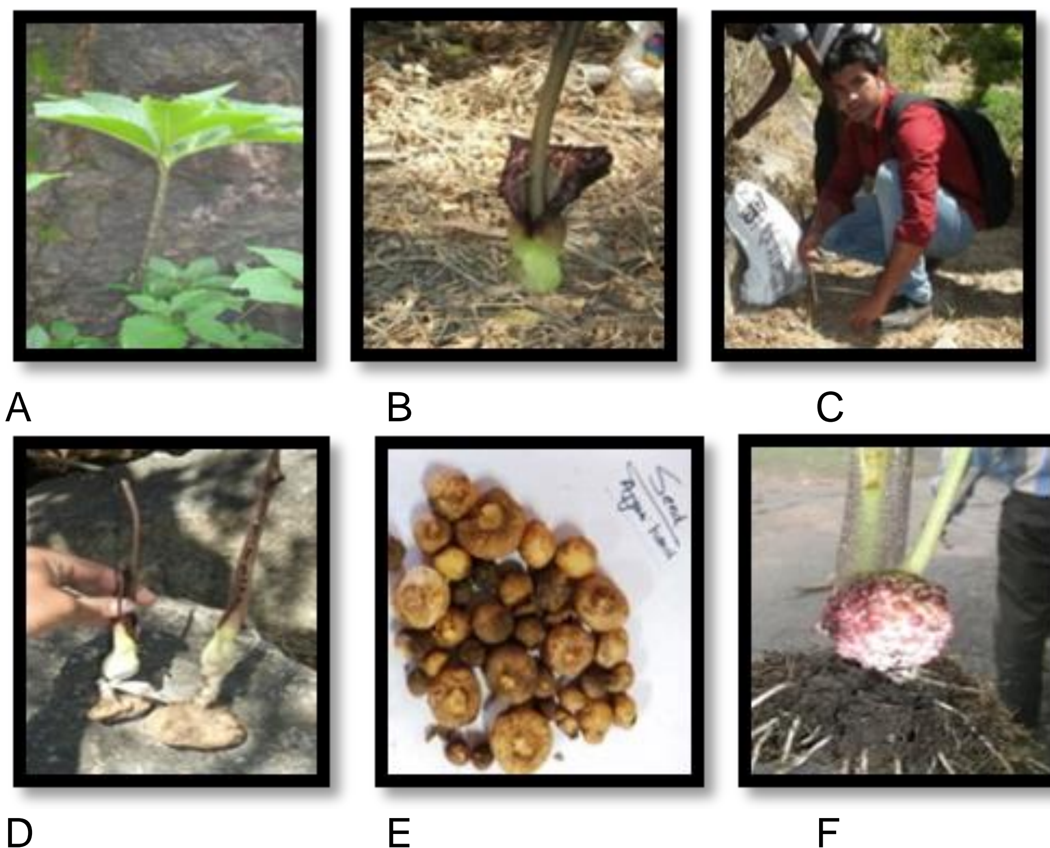

Figure 1. Different plant parts of *Amorphophallus konjac*. **A.** Whole plant; **B and D.** Mature stage. **C.** Research scholar collecting of plant material. **E.** Rhizome of plant. **F.** Root.

Amorphophallus is a genus of Aroid family and native to Asia. *Amorphophallus campanulatus sp.* showed antimicrobial efficacy against *Pseudomonas aeruginosa* and very less efficacy against *K. pneumonia* (Pandey and Gupta, 2013). *A. campanulatus* is used as fibrous diet in Indian food and locally known as *suran.* Ethnomedicinal background of *A. campanulatus* showed that it can cure snake bite which is used by tribal people of Rajasthan (Jain et al., 2005; Kavitha et al., 2011). *A. konjac* is also known as Snake Plant due to its morphology of snake like outlines on stem. It originates in South East Asia. They are recurrent plants with an alternative stem in the shape of a tuber and a highly dissect umbrella-shaped leaf sharp edge (Hetterscheid and Ittenbach, 1996). *Amorphophallus rivieri* is a synonym for *A. konjac* and it is commonly known as *devil's* tongue, snake palm or voodoo lily by local people. It has been cultivated in China for more than 2000 years (Long, 1998). Whole corm extract of this species have been used as a TCM for the treatment of asthma, cough, hernias, breast pain, burns and skin disorders (Niwa et al., 2010). Moreover, the corm tissues are known to be an important foundation of glucomannan, a soluble, non-cellulosic polysaccharide (Takigami, 2000).

Currently, konjac is grown in China, Japan, Korea, Indonesia and Thailand with a total crude flour manufacture more than 25,000 tones (Parry, 2010). China and Japan is the largest producer of konjac flour and account for 60 and 28%, respectively, of global manufacture. Konjac flour which has been reported as a laxative agent was konjac glucomannan that originated from Japan (*A. konjac*). Therefore, in the present research attempts were made to authenticate and validate the ethno-medicinal potentials of *A. konjac* antimicrobial activity which could be alternate for current synthetic antimicrobial agents. The plant was selected for screening of antimicrobial efficacy against selected eight bacterial strains. The results of antimicrobial activity of crude dicholormethane (DCM), ethyl acetate, chloroform and methanol were tested.

MATERIALS AND METHODS

Collection of plant material

A. konjac was collected in the month of April 2014 from tribal pockets of Mount Abu district Sirohi, Rajasthan, India (Figure 1). This plant was used by tribal's in their day to day lives to cure different ailments and commonly known as ajgarikand.

Identification

This sample were authenticated and was given identification number and submitted in Ethno-medicinal Herbarium, Centre with potentials of Excellence funded by DST, JECRC University, Jaipur, India. Further, voucher specimens of *A. konjac* was deposited at herbarium of University of Rajasthan, Jaipur, India and was verified by senior taxonomist of department and provided with accession no. RUBL211565.

Bacterial strains

Eight species of human pathogenic bacteria were obtained from Max Hospital Delhi in the month of October 2014. The colonies were authenticated with American Type Culture Collection (ATCC); bacterial species used for testing were *Staphylococcus aureus (ATCC- 2921), Klebsiella pneumoniae (ATCC 700603), Enterobacter cloacae (ATCC 13047), Escherichia coli (ATCC 25922), S. aureus (ATCC 25923), Proteus mirabilis (ATCC 12453), Enterobacter cloacae (ATCC- 13047), Enterococcus faecalis (ATCC 29212)* and *Streptococcus pneumoniae (ATCC 6305)* and were maintained on nutrient broth media.

Preparation of extracts

The tuber corms were washed well followed by exterior sterilization using 1% of sodium hypochlorite. The tubers were sliced into pieces, shade dried, and powered using an electric blender. Powdered crushed plant material (1 kg) of selected species was successively extracted with dicholormethane, ethyl acetate, chloroform, methanol and water. Extraction was done by Soxhlet apparatus using solvents in the increasing order of their polarity. Later, each of the homogenates was filtered and the residue was re-extracted twice for complete exhaustion, and the extracts were cooled individually. The obtained solvent extracts were concentrated using vacuum distillation process *in vitro* and re-dissolved in solvent, whenever screened for antimicrobial activity (Harborne, 1998).

Cultivation of test microorganisms

Bacteria was cultured on Nutrient agar medium (NAM). The medium was prepared using 20 g agar, 5 g peptone, 3 g beef extract and 3 g of NaCl in 1 L distilled water and sterilized at 15 lbs pressure and 121°C for 25 to 30 min. Agar test plates were prepared for pouring approximately 20 ml of NAM into the Petri dishes (10 mm) under aseptic laminar hood conditions. A peptone solution was prepared (by mixing 0.5% peptone) in distilled water, followed by autoclaving and the cultures were maintained on this medium by sub-culturing at regular interval with an incubation at 37°C for 24 to 48 h. For preparation of test plates, in bacteria, 10 ml of the respective medium was poured onto the Petri plates and used for screening.

Bactericidal assay

For bactericidal assay *in vitro* Disc diffusion method was adopted (Gould and Bowie, 1952), because of reproducibility and precision. The different test organisms were proceeded separately using a sterile swab over previously sterilized culture medium plates and the zone of inhibition was measured around sterilized dried discs of Whattman No.1 paper (6 mm in diameter), which contained three different concentration of respective solvent (A = 1 mg of test extract/disc; B = 5 mg of test extract/disc; C = 10 mg of test extract/disc) and tetracycline as reference drug (standard disc) separately. Further, treated discs were air dried at room temperature to remove any solvent, which may interfere with the strength, contaminations and inoculation. Initially the plates were exposed to low temperature for 1 h so as to allow the maximum diffusion of the compounds from the test disc into the agar plate and later, incubated at 37°C for 24 h in case of bacteria, after which the zone of inhibition could be made easily and calculated. Five replicates of each test extract were performed and the mean values were then referred. The zone of inhibition (IZ) in each result was recorded and the activity index (AI) was calculated respective to thestandard reference drugs (AI = Zone of inhibition of test sample / zone of inhibition of standard).

RESULTS AND DISCUSSION

A. konjac is known for its snake like stripes on stem and make its appearance unique in flora of Rajasthan. In Mount Abu the plant is grown in wild and known with some toxic principles. The results of antimicrobial activity of crude dicholormethane, ethyl acetate, chloroform and methanol are shown in Table 1. All the solvent extracts of Ajgarikand inhibited the growth of all the eight selected bacterial strains in a dose dependent manner. *Amorphophallus commutatus* was studied for morphological, phytochemical, and antibacterial properties (Krishna et al., 2013; Damle and Kotian, 2015), but till now systemic studies on the antimicrobial efficacy of *A. konjac* has not been studied so far. National Institute of Nutrition Standards recommended that *A. commutatus* possess all the edible part including macro and micro elements in adequate quantity (ICMR, 2009). Such studies provide a background for substitutes in ayurveda and provide a base for search of alternative plants from same family as nutraceuticals (Damle and Kotian, 2015; Pandey and Gupta, 2013). Therefore, to justify the ethno-medicinal use of *A. konjac* in ancient science, this scientifically validated the use.

Figure 2 shows various results of antimicrobial activity of crude dichloromethane (DCM), ethyl acetate, chloroform and methanol extracts. The results show that all the extract express appreciable efficacy against all the selected microorganisms. DCM extract C (10 mg/disc) possess maximum efficacy against *S. pneumoniae (*Inhibition zone = 20 mm; Activity Index = 1.25). The results show that efficacy of DCM extract are more than standard tetracycline. *S. pneumoniae* resides in nasopharynx of healthy carriers and cause community acquired pneumonia and meningitis resulted into coughing, sneezing etc. Therefore, use of DCM extract and bioactivity guided fractionation will lead to novel bioactives which will work as future antibiotics. Ethyl acetate extract also showed appreciable efficacy against the entire test organisms except *Proteus mirabilis* so using such extract in future can be used to isolate new chemical entity as broad spectrum drugs. It possess maximum efficacy against *S. aureus* (IZ = 18 mm; AI = 1.05) and *S. pneumoniae* (IZ = 25 mm; AI = 1.56). It is also noteworthy that the minimum inhibition concentration of both the ethyl acetate disc also possess efficacy

Table 1. Antibacterial activity of dichloromethane, ethyl acetate, chloroform and methanol extracts of *A. konjac*.

Solvent type	Extract		EC	SA	KP	SAB	PM	ECL	EF	SP
Standard (Tetracycline)		IZ	17	17	19	19	11	18	15	16
Dicholoro-methane	A	IZ	-	-	-	6	-	6	5	16
		AI	-	-	-	0.31	-	0.33	0.33	1
	B	IZ	-	5	-	7	-	6	6	13
		AI	-	0.29	-	0.36	-	0.33	0.4	0.81
	C	IZ	6	6	-	8	-	7	7	20
		AI	0.35	0.35	-	0.42	-	0.38	0.46	1.25
Ethyl acetate	A	IZ	5	16	5	6	-	8	6	23
		AI	0.29	0.94	0.26	0.31	-	0.44	0.4	1.43
	B	IZ	6	17	9	8	-	10	7	24
		AI	0.35	1	0.47	0.42	-	0.55	0.46	1.5
	C	IZ	7	18	11	6	-	10	8	25
		AI	0.41	1.05	0.57	0.31		0.55	0.53	1.56
Chloroform	A	IZ	6	-	8	6	-	6	5	6
		AI	0.35	-	0.42	0.31		0.33	0.33	0.37
	B	IZ	7	6	9	7	-	7	9	11
		AI	0.41	0.35	0.47	0.36		0.38	0.6	0.68
	C	IZ	9	8	9	8	-	8	8	9
		AI	0.52	0.47	0.47	0.42		0.44	0.53	0.56
Methanol	A	IZ	-	-	-	-	-	6	5	10
		AI	-	-	-	-	-	0.33	0.33	0.62
	B	IZ	-	6	-	6	6	6	6	-
		AI	-	0.35	-	0.31	0.54	0.33	0.4	-
	C	IZ	7	7	-	7	6	9	8	12
		AI	0.41	0.41	-	0.36	0.54	0.5	0.53	0.75

I.Z. = Inhibition zone showed by extract against microorganism in mm; AI = Activity index of extract; SA = *Staphylococcus aureus* (ATCC- 2921); KP = *Klebsiella pneumoniae* (ATCC 700603); EC = *Escherichia coli* (ATCC 25922); SAB = *Staphylococcus aureus* (ATCC 25923); PM = *Proteus mirabilis* (ATCC 12453); ECL = *Enterobacter cloacae* (ATCC- 13047); EF = *Enterococcus faecalis* (ATCC 29212); SP = *Streptococcus pneumonia* (ATCC 6305); - = no inhibition zone; A = 1 mg/disc; B = 5 mg/disc; C = 10 mg/disc.

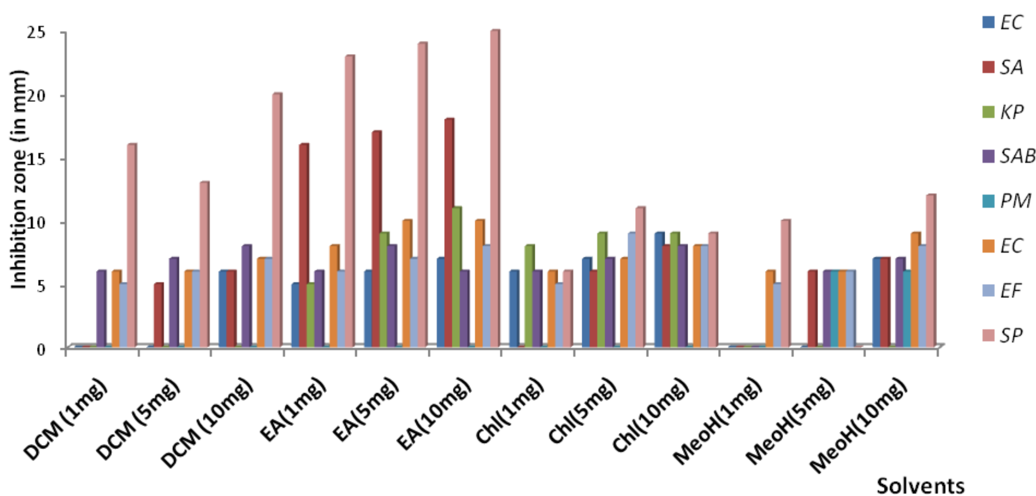

Figure 2. Antibacterial activity of dichloromethane, ethyl acetate, chloroform and methanol extracts of *A. konjac*. SA = *Staphylococcus aureus* (ATCC- 2921); KP = *Klebsiella pneumoniae* (ATCC 700603); EC = *Escherichia coli* (ATCC 25922); SAB = *Staphylococcus aureus* (ATCC 25923); PM = *Proteus mirabilis* (ATCC 12453); ECL = *Enterobacter cloacae* (ATCC- 13047); EF = *Enterococcus faecalis* (ATCC 29212); SP = *Streptococcus pneumonia* (ATCC 6305).

more than standard which is really a remarkable feature.

Further, processing of this extract will lead to generation of new antibiotics. Chloroform and methanol extracts showed significant efficacy against all the test microorganisms whereas the maximum efficacy against *S. pneumoniae* (IZ = 11 mm; AI = 0.68) and (IZ = 12 mm; AI = 0.75), respectively. The main cause of community acquired pneumonia and septicemia in HIV infected patients is caused by *S. pneumoniae*. Further, bioactivity guided fractionation from DCM extract of *A. konjac* can lead to isolation of pure compounds as novel antibiotic in future. Therefore, the extract can also be used for isolation of volatiles compounds with potentials so that the extract/ active fraction/ pure compounds can be used as nasal spray in future therapeutics.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Lipid lowering and anti-atherosclerotic properties of *Tinospora crispa* aqueous extract on high-cholesterol diet-induced hyperlipidemic rabbits

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This study was aimed to investigate the hypocholestrolemic and anti-atherosclerotic properties of *Tinospora crispa* aqueous extract (TCAE) on rabbits for 10 weeks. The hyperlipidemic rabbits were induced and the rabbit were given different concentration of TCAE (200, 450 and 600 mg/kg). Results from lipid analysis show that the level of total cholesterol (TC), triglyceride (TG) and LDL-C on the hyperlipidemic rabbits were reduced with the treatment of TCAE while HDL level was elevated. Through plasma analysis, the activity of gamma glutamyl transferase (GGT) and alkaline phosphates (ALP) were also reduced with the treatment of TCAE compared to hyperlipidemia group. All group of rabbits tested with TCAE again had significantly higher ($p < 0.05$) total antioxidant status (TAS), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. Among the concentrations of TCAE tested, medium dose showed more potent effect in reducing blood serum TC, TG and LDL-C levels and increasing HDL-C level compared to low and high dosages counterparts. No foam cell formation was visible in aorta of rabbits treated with TCAE in dose dependent manner. However, there was visible foam cell formation in the aorta of hyperlipidemia group. In conclusion, this study suggests that supplementation of 450 mg/kg of *T. crispa* extract would be able to reduce or retard the progression of atherosclerotic plaque development induced by dietary cholesterol.

Key words: Hypocholesterolemia, *Tinospora crispa*, anti-atherosclerotic properties, cardiovascular diseases.

INTRODUCTION

Cardiovascular diseases (CVD) is a world's largest killer, claiming 17.5 million lives a year in 2012, representing 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke (WHO, 2015). In Malaysia, cardiovascular related diseases contribute to 24.38% of reported death in government hospital (MOH, 2014). Hyperlipidemia is one of contributing factor for cardiovascular diseases (CVD) such as atherosclerosis, stroke and myocardial infarction (Kannel et al., 1979; Xing et al., 2009).

Hyperlipidemia refers to increased level of total cholesterol (TC), triglyceride (TG) and low density lipoprotein (LDL) as well as decreased level of high density lipoprotein (HDL). Reducing blood TC, TG and LDL level, while increasing blood HDL level has become beneficial factor to overcome CVD, thus evoke many investigators to design drugs with the ability to manage blood cholesterol level. Among them, statin has become the most popular kind of drug that has been produced. However, there are many side effects that have been reported on this drug consumption including myotoxicity and liver disfunction (Sathasivam, 2012). Herbal remedies have become popular in recent decades as an alternative treatment over modern medicines in the screening of hyperlipidemic cure. According to WHO, 80% of the population in some Asian and African countries depend on traditional and herbal medicine for primary health care (WHO, 2002). There are several studies on the therapeutic effect of herbal extracts on various kinds of diseases and ailments including diabetes, hypertension and CVD. There are also many study performed previously which showed that herbal extracts can reduce lipid level on hypercholesterolemia rabbit (Berger et al., 2004; Chen et al., 2011; Khanna et al., 2002).

Tinospora crispa, plant locally known as patawali in Malaysia is a climber that can be found in primary rainforest widely distributed in Malaysia, Indonesia, Thailand and Vietnam. To date, several activity of *T. crispa* has been recorded including anti-diabetic (Noor and Ashcroft, 1998), hypolipidemia (Praman et al., 2011) and anti-inflammatory (Sulaiman et al., 2008). The aqueous extract of *T. crispa* stem can be taken to treat diabetes mellitus and improve diabetic conditions (Noor and Ashcroft, 1998). Traditionally, this plant was found to be used to treat diabetes, hypertension and lumbago (Fasihuddin and Hasmah, 1991). The stems of *T. crispa* were commonly used to treat coughs, asthma, fever and stomach acnes (Perry, 1980; Muhammad and Mustafa, 1994). Other than that, it also can be used for purifying blood and as preventions against bacterial and viral infections (Quisumbing, 1978). Besides, the young stems of the plant can be taken raw to reduce high blood pressure, diabetes and relieve abdominal pains (Ministry of Industry and Primary Resources of Brunei Darussalam, 1992).

Present study sought to investigate the lipid lowering properties of *T. crispa* aqueous extract (TCAE) on the hyperlipidemic rabbit model and the parameters of hyperlipidemia including TC, TG, LDL and HDL were investigated. On the other hand, several biochemical parameters that are related to the CVD occurrence such as SOD and GPx antioxidant enzyme levels and liver function enzymes such as GST and ALP were also measured.

MATERIALS AND METHODS

Preparation of *T. crispa* extract

Fresh stems of *T. crispa* were collected from Forest Research Institute Malaysia (FRIM) at Kepong, Selangor after proven by plant taxonomist. The stems were then cut into small pieces of around 2 inches long followed by drying in the oven at 55°C for 72 h. The completely dried stems were then ground into powder by using bench-top blender. *T. crispa* aqueous extract was prepared by adding 100 g *T. crispa* powder to 1000 ml distilled water and shaken under shaking water bath at 55°C for 4 h. The solution was then filtered and the supernatant was freeze dried at -80°C. The finished processed of *T. crispa* aqueous extract in powder form was then store at -20°C until use.

Induction of experimental hyperlipidemic rabbits

Forty two (42) male New Zealand White (NZW) rabbits with initial mean body weight of 2.5 to 3.0 kg were acclimatized under room temperature (28 ± 2°C) with a regular light/dark cycle and free access to food and water for 2 weeks before use. Following acclimatization, the animals were randomly segregated into six groups of seven rabbits each. Normal control (NC) group was given normal chow diet while other 5 groups were given 0.5% cholesterol chow diet for continuous 10 weeks to induce hyperlipidemic rabbit model. Food and water were given *ad libitum* throughout the experiment. Then, the 5 groups were assigned as hyperlipidemia group (H), simvastatin group (SC), low dosage (200 mg/kg) of TCAE, medium dosage (450 mg/kg) of TCAE and high dosage (600 mg/kg) of TCAE. The TCAE and simvastatin were given via oral gavage while blood sampling was performed 10 weeks of experimental period. The experimental protocol and animal handling throughout the study were in accordance with guidelines approved by the institution ethics committee where the study is conducted.

Biological sample collection

The animal was sacrificed by exsanguinations for blood and organ collection after 12 h of fasting using diethyl ether as anesthetic. Approximately 5 mL of blood was collected by cardiac puncture, segregated into lithium heparin and EDTA tubes. The bloods collected in EDTA tubes were centrifuged at 3000 rpm for 10 min at

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Abbreviations: TCAE, *Tinospora crispa* aqueous extract; **GGT,** gamma glutamyl transferase; **ALP,** alkaline phosphates; **TAS,** total antioxidant status; **GPx,** glutathione peroxidase; **SOD,** superoxide dismutase; **CVD,** cardiovascular diseases; **TC,** total cholesterol; **TG,** triglyceride; **HDL-c,** high density lipoprotein-cholesterol; **LDL-c,** low density lipoprotein-cholesterol; **GSH-Px,** glutathione peroxidase; **VLDL,** very low density lipoprotein; **IDL,** intermediate density cholesterol; **ROS,** reactive oxygen species.

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4°C to separate the plasma. The serum was then transferred into eppendorf tubes, labeled, covered with aluminium foil and stored at -80°C until further analysis. The blood serum was used for estimation of lipid profiles, liver toxicity enzymes and total antioxidant status (TAS). The bloods collected in heparin tube were centrifuged at 3000 rpm for 10 min at 4°C to separate the plasma from the erythrocyte. The plasma was then removed and erythrocyte was washed with sodium chloride (0.9 w/v NaCl) which was centrifuged at 3000 rpm for 10 min at 4°C. The erythrocyte was washed with sodium chloride and repeated for three times. Before analysis, sodium chloride was added to the erythrocyte.

Lipid analysis

Lipid analysis such as total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-c) and low density lipoprotein-cholesterol (LDL-c) in serum were determined by enzymatic methods using Rosche commercial kit (Germany) in accordance with the manufacturer instruction. All the serums were measured using Hitachi Chemistry Analyzer 902 Machine at the Chemistry Pathology Laboratory, Faculty of Medicine and Health Sciences, University Putra Malaysia. The test utilizes the principle of enzymatic colorimetric assay to read the sample.

Antioxidant enzyme test

Antioxidant enzyme activities of glutathione peroxidase GPx, superoxide dismutase (SOD) as well as total antioxidant status (TAS) were determined in the whole blood using a kit of Randox in accordance of manufacturer instruction. All the serums were measured using Cobas Mira Plus analyzer at the Research Laboratory of Faculty of Medicine and Health Sciences, UPM.

Plasma liver function test

The metabolic processes and variety of chemicals that occur in the liver were maintained by the help of liver enzymes including Alkaline Phospatase (ALP) and Gamma Glutamyl Transpeptidase (GGT). In this study, these three enzymes were used to monitor the liver toxicity caused by the treatment effect. The level of ALP and GGT was measured using Roche commercial kit in accordance with the manufacturer instruction. All the serums were measured using Hitachi Chemistry Analyzer at the Chemistry Pathology Laboratory, Faculty of Medicine and Health Sciences, University Putra Malaysia. All enzymes of interest were measured using the enzymatic colorimetric test principle.

Histology study

After the rabbits were killed, aorta tissue between its origin and bifurcation into the iliac arteries was taken gently, free of adhering tissues and washed with cold normal saline solution. The large part of origin was cut into 2 mm^3, and was put into 10% formalin for haematoxylin and eosin staining, whereas the ascending large part of aorta between its origin and bifurcation into the iliac arteries was opened longitudinally and prepared for plaque assay.

Haematoxylin and eosin staining

The aortic arch were fixed in 10% formalin for a few days and prepared for light microscopy by dehydrating the tissue samples in an ascending series of alcohol dehydration, clearing with xylene and wax impregnation with paraffin wax for 14 h in an automatic tissue processor machine. The tissues were then embedded into block by paraffin wax at 62°C and was cooled at 0°C for 3 h to form solid block. This is followed by the sectioning process, whereas the tissues were trimmed and sectioned with the thickness of 4 to 5 μm using a microtome machine. The tissues were then placed in the water bath, attached on glass slides and then were dried on a hot plate at 50 to 55°C for 30 min and then kept at 37°C. The tissues sections were then stained with Haematoxylin and Eosin (H&E) staining method using Autostainer Machine. The slides underwent processing, colorization and dehydration. After thoroughly dried from xylene, the slides were mounted with cover slips and mounted with DPX. The slides were then dried at room temperature for a few days before being analyzed by an Image Analyzer Machine. The aortic arch aorta was evaluated quantitatively.

Assessment of atherosclerotic plaque lesions

Atherosclerotic plaque areas were assessed by a previously described method (Prasad and Kalra, 1993). Briefly, the aortic strips were dissected from the ascending arch to the iliac bifurcation, and extraneous adipose tissue was removed. The aortas were opened longitudinally, rinsed several times with ice-cold saline and stretched onto a piece of cardboard. Then, it was fixed immersed in neutral 100 g/L buffered formalin solution for 24 h and then rinsed in 70% alcohol. The tissue was then immersed in Herxheimer's solution containing Sudan IV (5 g), ethyl alcohol (70%, 500 mL) and acetone (500 mL) at room temperature for 15 min and washed in running water for 1 h. Photographs of the intimal surface of the aorta were taken using digital camera (EOS Canon, Japan) and the intimal lipid lesions were determined quantitatively by estimation of the percentage of sudanophilic stained areas in the total aortic intimal area in photographs using Image Analysis Software. The total atherosclerotic area of the intimal surface of the aorta was measured in mm^2. The extent of atherosclerosis was expressed as a percentage of the luminal surface that was covered by atherosclerotic plaques.

Statistical analysis

Statistical analysis for all assays were performed by one-way ANOVA with Dunnett's posthoc multiple group comparison using GraphPad Prism software (Version 5). $P<0.05$ and $P<0.01$ was considered significant for all tests.

RESULTS

Hypolipidemic effect of TCAE in hyperlipidemic rabbits

Hypolipidemic effect of TCAE and simvastatin are shown in Table 1. When compared to control (normal rabbits), TC, TG and LDL-C and HDL-C level were markedly increased in hyperlipidemic rabbit. After the treatment of TCAE at 200, 450 and 600 mg/kg and simvastatin, there were significant decrease of TC, TG and LDL-C level in serum lipid. TC level of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin decreased by 35.89, 50.38, 33.94 and 95.03%, respectively, compared to untreated hyperlipidemia group while TG level of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin decreased by 73.86, 77.27, 60.23 and 78.98%,

Table 1. Effect of TCAE and simvastatin on the level of TC, TG, LDL-C and HDL-C in rabbit blood serum following 10 weeks of treatment.

Treatment group	Serum lipid profile (mmol/L) (mean ± SD)			
	TC	TG	LDL-C	LDL-C
NC	0.96±0.02*,#	0.36±0.02*,#	0.16±0.02*,#	0.59±0.06
H	29.20±1.54	1.76±0.18	25.72±0.25	0.97±0.48
Simvastatin	1.45±0.74*,#	0.37±0.03*,#	1.43±0.45*,#	1.86±0.37*,#
TCAE (mg/kg)				
200	18.72±0.37*,#	0.46±0.04*,#	14.50±0.34*,#	4.42±0.26*,#
450	14.49±1.83*,#	0.40±0.02*,#	12.57±0.28*,#	5.85±0.92*,#
600	19.29±1.03*,#	0.70±0.02*,#	15.68±0.25*,#	4.53±0.24*,#

Each value represents the mean ± SD. Values with the asterisk (*) are significantly different ($p < 0.05$) compared to hyperlipidemia model group in respective test group. Values with # are significantly different ($p < 0.05$) compared to normal control group in respective test group. TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein; LDL-C, high density lipoprotein; NC, normal control; H, high cholesterol diet group; TCAE, *T. crispa* aqueous extracts.

Table 2. Percentage of atherosclerotic plaques coverage of all groups.

Parameter	TCAE (mg/kg)					
	NC	H	Simvastatin	200	450	600
% of coverage	0.00±0.00*,#	31.12±2.18	2.24±0.35*,#	25.42±0.60*,#	13.14±0.63*,#	15.29±0.57*,#

Each value represents the mean ± SD. Values with the asterisk (*) are significantly different ($p < 0.05$) compared to hyperlipidemia model group. Values with # are significantly different ($p < 0.05$) compared to normal control group. NC, normal control; H, high cholesterol diet group; TCAE, *T. crispa* aqueous extracts.

respectively, when compared to untreated hyperlipidemia group. LDL-C level of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin decreased by 43.62, 51.12, 39.04 and 94.44%, respectively, while LDL-Clevel of hyperlipidemic rabbits treated with low, medium, and high dosages of TCAE markedly increased by 4.55, 6.22 and 4.67 fold, respectively, compared to untreated hyperlipidemia group.

Effect of TCAE on atherosclerotic plaques coverage

Atherosclerotic plaques coverage of hyperlipidemic rabbit, treatment group and simvastatin was measured using NC as a baseline. Hyperlipidemia rabbit showed marked increase of plaques coverage with 31.12 ± 2.18% of coverage. TCAE showed marked reduction of atherosclerotic plaques coverage when compared to hyperlipidemic rabbit and the reduction of plaques area was 18.32, 50.87 and 66.25% of reduction for 200, 450 and 600 mg/kg of TCAE, respectively (Table 2). Simvastatin reduced plaques coverage to a greater extent with 92.80% of reduction.

Effect of TCAE on liver enzymes expression

Effect of TCAE on liver enzymes expression is shown in Figure 1. Compared to control (normal rabbits), GGT expression on hyperlipidemic rabbits significantly increased while there was no significant difference in ALP expression between hyperlipidemic rabbit and normal rabbit. Treatment of TCAE at 200, 450 and 600 mg/kg and simvastatin significantly decreased GGT and ALP level, when compared to hyperlipidmic rabbit. GGT level of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin were declined by 66.63, 77.81, 76.00 and 14.42%, respectively, compared to untreated hyperlipidemia group. ALP level of hyperlipidemic rabbit treated with low, medium, high dosages of TCAE and simvastatin decreased by 76.64, 89.38, 83.72 and 71.78%, respectively, compared to untreated hyperlipidemia group

Effect of TCAE on antioxidant enzymes activity and total antioxidant status

Effect of TCAE on antioxidant enzymes activity and total antioxidant status is shown in Figure 2. Compared to those of rabbit in control group (normal rabbits), SOD and GPx activity as well as TAS of hyperlipidemic rabbits was significantly reduced. Treatment of TCAE at 200, 450 and 600 mg/kg and simvastatin significantly increased SOD and GPx activity as well as TAS when compared to hyperlipidmic rabbits. SOD activity of hyperlipidemic

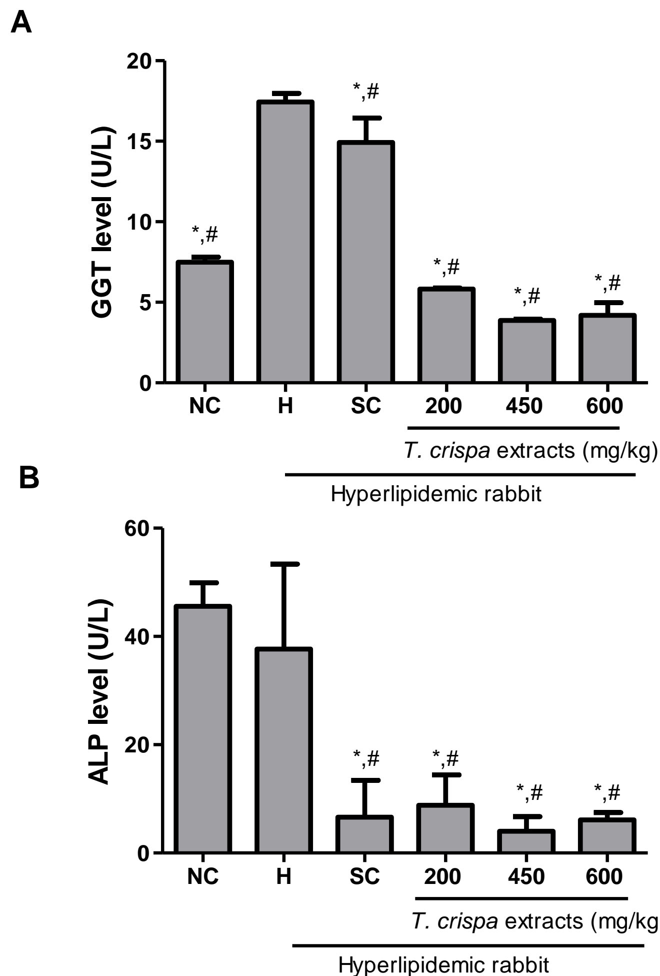

Figure 1. Effect of TCAE and Simvastatin on the level of A) GGT and B) ALP level of all Groups. Each value represents the mean ± SD. Values with the asterisk (*) are significantly different (p<0.05) compared to hyperlipidemia model group in respective test group. Values with # are significantly different (p<0.05) compared to hyperlipidemia group in respective test group. GGT, gamma glutamyl transpeptidase ; ALP, alkaline phosphatase level; NC, normal control; H, high cholesterol diet group.

rabbits treated with low, medium, high dosages of TCAE and simvastatin were increased by 4.22, 7.64, 8.30 and 1.48 fold, respectively, compared to untreated hyperlipidemia group while GPx activity of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin were increased by 1.65, 1.60, 1.65, 1.87 and 4.48 fold, respectively, compared to untreated hyperlipidemia group. TAS of hyperlipidemic rabbits treated with low, medium, high dosages of TCAE and simvastatin were not signicantly altered compared to hyperlipidemic rabbits.

DISCUSSION

Hyperlipidemia is a major pathological basis of CVD such as atherosclerosis, stroke and myocardial infarction.

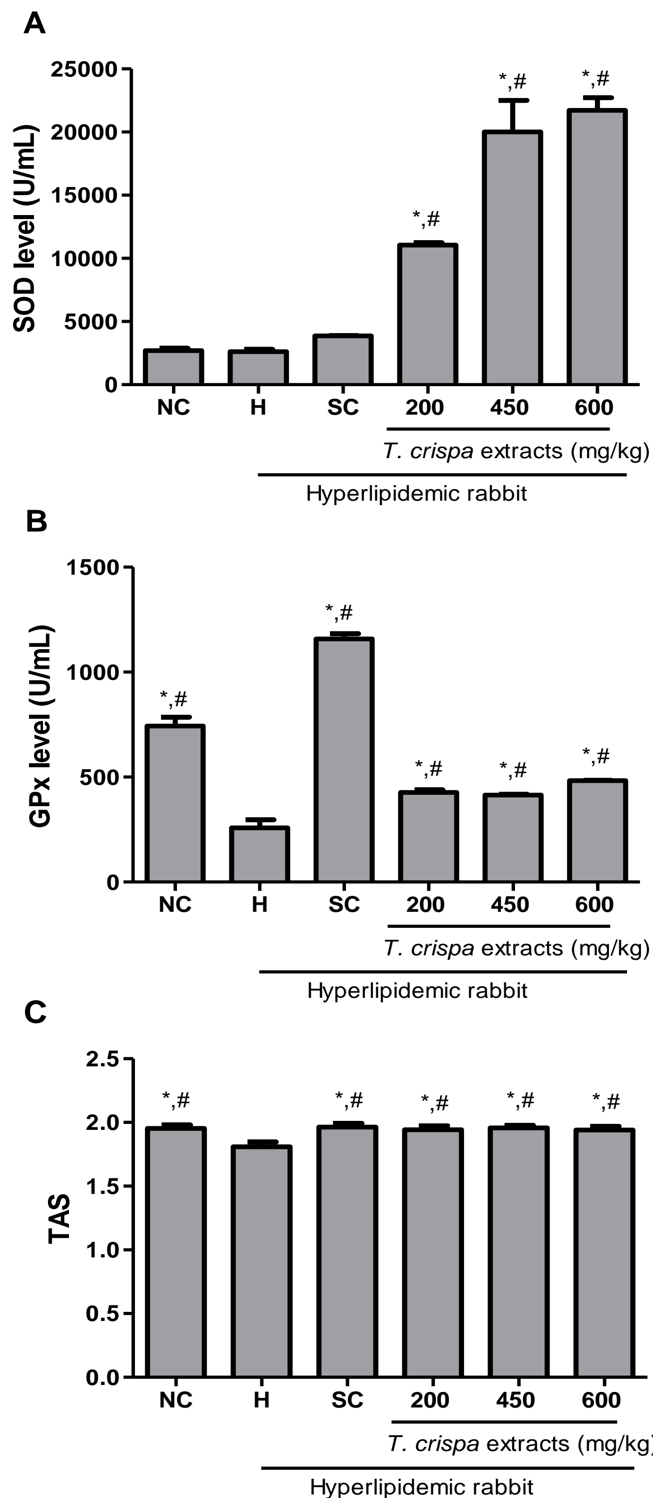

Figure 2. Effect of TCAE and Simvastatin on the level of A) SOD level, B) GPx Level and C) TAS of all Groups. Each value represents the mean ± SD. Values with the asterisk (*) are significantly different (p<0.05) compared to hyperlipidemia model group. Values with # are significantly different (p<0.05) compared to normal control group. SOD, superoxide dismutase; GPx, glutathione peroxidase; TAS, total antioxidant status; NC, normal control; H, high cholesterol diet group; TCAE, *T. crispa* aqueous extracts.

Hyperlipidemia, particularly hypercholesterolemia, can increase the risk factor of atherosclerosis by inducing arterial endothelial dysfunction (Chen et al., 2011). LDL also has been diagnosed to be a major determinant to the hypercholesterolemia. Different from LDL, HDL plays a role in reverse transport of cholesterol from artery back to the liver at which it is eliminated. Therefore, managing the elevation of blood LDL, TG, and TC levels are important to prevent HL and atherosclerosis, and are of great significance in reduction of CVD incidence. Result of this study shows that, following a week of acclimatization, there were no significant differences in blood parameters of all rabbits. However, rabbits fed with high cholesterol diet over 10 weeks showed significantly higher level of serum TC, TG and LDL-C compared to normal diet rabbits. This result showed that the hyperlipidemic rabbit model have been established after been introduced to high cholesterol diet over 10 weeks period. The levels of TC, TG and LDL-C on these hyperlipidemic rabbits however, were reduced when it was administered to different dosages of TCAE. Among the concentrations of TCAE tested, medium dose showed more potent effect in reducing blood serum TC and LDL-C levels compared to low and high dosages counterparts. For blood serum TG level, low and medium dosages of TCAE exhibit more potent effect to reduce blood serum TG level, compared to high dosage of TCAE. In addition to that observed result in LDL-C, HDL-C and TC, TCAE also reduced the coverage area of atherosclerotic plaque compared to hyperlipidemic rabbits group in dose dependent manner. It is well known that increased level of TC, TG and LDL-C were the major symptom of hyperlipidemia. Hyperlipidemia, in turn is a major risk factor of atherosclerosis (Nelson, 2013). The decrease of TC, TG and LDL-C level of hyperlipidemia group after treatment of TCAE have showcase the beneficial effect of TCAE consumption in reducing serum cholesterol level and these results have been supported by the reduction of atherosclerotic plaque coverage of hyperlipidemic rabbit that were treated with TCAE. Total cholesterol refers to total amount of cholesterol at a given time and the sum of LDL-C, HDL-C, very low density lipoprotein (VLDL) and intermediate density cholesterol (IDL). In this study, TC was significantly increased in hyperlipidemic rabbit compared to untreated rabbit. However, TCAE at different dosage evidently decrease the TC level and the medium dosage of TCAE has again showed the most evident effect.

Gamma glutamyl transferase (GGT) is an enzyme which plays a role in extracellular catabolism of antioxidant glutathione. It can be found in liver, kidney and cerebrovascular endothelium. GGT acts as pro-oxidant in extra-cellular space. The increase level of GGT may be a reflection of high degree of oxidative stress. GGT is also found to be positively correlated with increase of chronic heart disease events such as congestive heart failure and components of the metabolic syndrome (Niranjan et al., 2012). Results of GGT test in this study shows that TCAE has markedly reduced GGT level on hyperlipidemic rabbit. GGT level in rabbit administrated with medium and high dosages of TCAE decreased by 77.81 and 76.00%, respectively, compared to hyperlipidemic rabbits model. Even though low dosage of TCAE showed lower inhibitory effect than medium and high dosages, it also produced a substantial inhibitory effect with 66.63% of inhibition. Alkaline phosphatase (ALP), a membrane bound isoenzymes that catalyzes the hydrolysis of inorganic pyrophosphate, is expressed in variety of tissues including liver and bone, and in lesser amounts from intestines, placenta, kidneys, and leukocytes (Tonelli et al., 2009). Previous epidemiological studies have associated serum ALP levels to increased coronary calcification and increased risk of CVD (Adeney et al., 2009; Covic et al., 2009; Palmer et al., 2011) and the proposed mechanism in which ALP is related to CVD may be due to inflammation (Tonelli et al., 2009). In this study, medium dosage of TCAE has again showed a strong inhibition activity compared to low dosage counterpart while it is not significantly different compared to high dosage counterpart. The ability of TCAE to inhibit the release of GGT and ALP marker demonstrate its protective effect from oxidative stress and prevent coronary calcification thus decrease the risk of CVD.

Oxidative stress, apart from hyperlipidemia and liver biomarkers, is also an important risk factor for atherosclerosis and CVD. Many reported studies has shown that oxidative stress cause oxidation of LDL to ox-LDL and cause endothelial cell injury which lead to infiltration of monocyte to the arterial intima and its deposition into arterial membrane. Oxidation of LDL to ox-LDL is mainly caused by reactive oxygen species (ROS), particularly superoxide anion and hydrogen peroxide (H_2O_2) (Liu et al., 2009). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are intracellular enzymes that are involved in cellular defence mechanism from oxidative stress caused by ROS. SOD plays a role in scavenging superoxide anion by forming H_2O_2 while GPx safely decompose H_2O_2 to water and superoxide anion (Yu et al., 2007). In normal circumstances, both enzymes can steadily eliminate oxidative stress by feedback compensatory mechanism. However, under excessive oxidative stress condition caused by superoxide anions, or with limited bioavailability of endogenous antioxidants, both enzymes were depleted and it is reflected by low level of SOD and GPx in plasma. In corroboration with that, result of this study shows that GPx level in hyperlipidemic rabbits was reduced when it was compared to normal rabbit group. Rabbits that were treated with TCAE exhibited increased level of SOD and GPx compared to hyperlipidemic rabbits and even at higher level compared to normal group, demonstrating the protective effect of TCAE in regulating antioxidant enzymes level under oxidative stress circumstance.

In this case, the increased level of SOD and GPx on TCAE-treated rabbits was in dose dependent manner, which contradicts the results of the aforementioned experiments. This result demonstrates the antioxidant capability of TCAE by increasing endogenous antioxidant enzymes on hyperlipidimic rabbits.

Conclusion

These findings suggest that treatment of TAEC were able to positively modulate cholesterol metabolism. Supplementation of 450 mg/kg of *T. crispa* extract would be the optimum concentration to reduce or retarde the progression of atherosclerotic plaque development induced by dietary cholesterol by reducing LDL-C, TC, TG, ALP and GGT and raising HDL-C, TAS, GPx and SOD. *T. crispa* may, therefore, be beneficial in preventing hypercholesterolemic, atherosclerosis and reducing risk factors for coronary artery disease.

Conflict of interests

The authors did not declare any conflict of interest.

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The inhibitory effect of *Lactobacillus sakei* KBL isolated from kimchi on the adipogenesis of 3T3-L1 cells

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Abnormal adipocyte growth, in terms of increased cell numbers and increased cell differentiation, is considered to be a major pathological feature of obesity. Thus, the inhibition of preadipocyte mitogenesis and differentiation could help prevent and suppress obesity. Some probiotics and cellular components are known to modulate the lipid metabolism *in vitro* and/or *in vivo*. The aim of this study was to investigate whether extracts from *Lactobacillus sakei* KBL cells isolated from kimchi could exert anti-adipogenic action in 3T3-L1 cells (fat cells). Differentiating 3T3-L1 cells were treated with *L. sakei* KBL cell extracts (*L. sakei* KBL_CE), and cell viability was assessed by MTT assays. At concentrations below 1 mg/ml, *L. sakei* KBL_CE did not exert any cytotoxic effect in 3T3-L1 cells. Lipid regulation in the cell culture system was assessed by morphological analysis and oil-red-O staining of fat. Treatment with *L. sakei* KBL_CE significantly inhibited adipocyte differentiation. *L. sakei* KBL_CE treatment (1 mg/ml) also reduced lipid accumulation by 25% in fully differentiated 3T3-L1 adipocytes. These findings collectively indicate that *L. sakei* KBL_CE can reduce fat mass by modulating adipogenesis in maturing preadipocytes.

Key words: *Lactobacillus sakei* KBL, 3T3-L1 cells, adipocyte differentiation, obesity.

INTRODUCTION

Probiotics are defined as viable microorganisms that when consumed in adequate amounts, confer health benefits by improving the properties of indigenous microflora (Guillot, 1998). These effects include the amelioration of hypercholesterolemia (Park et al., 2007) and hypertension (Aihara et al., 2005), prevention of cancer (Rafter, 2004), modulation of the immune system (Baken et al., 2006) etc. According to a recent study, live probiotics, dead probiotic cells, and even probiotic cell components can exert significant biological effects outside the gastrointestinal tract (Adam, 2010). Thus, probiotics are believed to be an important part of an overall dietary strategy for maintaining health. However, the identification of various probiotic strains and their mechanisms of action are not fully elucidated, yet. Some probiotics have been found to be effective in regulating

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Abbreviations: *L. sakei* KBL_CE, *Lactobacillus sakei* KBL_cell extract; **FBS,** fetal bovine serum; **PS,** penicillin-streptomycin; **DMEM,** dulbecco modified eagle medium; **IBMX,** 3-isobutyl-1-methylxanthine; **MDI,** 3-iso-butyl-1-methylxanthine, dexamethasone and insulin.

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adipose tissue in overweight adults and animal models of obesity. In particular, administration of *Lactobacillus plantarum* to mice led to reductions in adipose tissue weights (Sato et al., 2008; Ma et al., 2008).

Obesity, which occurs when excessive fat accumulates due to an overage of energy intake compared to consumption, is associated with various health consequences, such as cardiovascular disease, diabetes mellitus, and other chronic disorders (Spiegelman and Flier, 2001; Macelin and Chua, 2010). Obesity is widely recognized as a worldwide problem due to its negative health consequences and huge cost to society. Thus, we critically need safe and effective anti-obesity measures. Despite this urgent need and the potential size of the market for anti-obesity drugs, however, the developed drugs have proven unsatisfactory to date, due to their deleterious side effects. Thus, some researchers are turning to edible natural products that have 'historically' been used as dietary supplements for body-weight management and control in many countries (Rayalam et al., 2008). Obesity primarily arises via increased cytoplasmic triglyceride deposition, which leads to adipocyte enlargement, and elevated adipogenesis, which results in the formation of new adipocytes from precursor cells (Gregoire, 2001). Adipose tissue has been shown to secrete a variety of bioactive peptides (that is, adipokines) that can potentially affect glucose and lipid metabolism (Spiegelman and Flier, 2001). As adipocyte differentiation and the extent of subsequent fat accumulation are closely related to the occurrence and advancement of various diseases, inhibiting the proliferation and differentiation of fat cells is considered to be an important strategy for the potential treatment of obesity (Rayalam et al., 2008; Rosen and Spiegelman, 2006). In many obesity-related studies, the fibroblastic 3T3-L1 preadipocyte line is widely used to investigate the mechanism of preadipocyte proliferation and adipocyte genesis, due to the ability of this cell line to undergo complete differentiation into mature adipocytes.

Kimchi is a fermented cabbage product that is traditional in the Republic of Korea (Chang et al., 2010). Among the lactic acid bacteria (LAB; a subset of probiotic organisms), isolated from kimchi, *Lactobacillus sakei* is a psychrophilic bacterium that is found as the dominant species in kimchi produced at -1°C (Cho et al., 2006). *L. sakei* raises interest on anti-obesity LAB. However, there is little information available on the anti-obesity effects of *L. sakei* LAB. Here, we investigated the effects of *L. sakei* KBL cell extracts on the cell viability and intracellular lipid accumulation in cultured and differentiating 3T3-L1 (adipocyte) cells.

MATERIALS AND METHODS

Isolation of *L. sakei* KBL and preparation of the cell extract

L. sakei KBL was isolated from kimchi and confirmed by DNA sequencing of 16S rRNA. For experiments, *L. sakei* KBL was grown anaerobically in deMan-Rogosa-Sharpe (MRS) medium at 37°C for 18 h, and then the cells were collected by centrifugation and washed with phosphate-buffered saline (PBS). The cells were counted and suspended in PBS at 10^{10} colony-forming units (CFU)/ml, and then subjected to sonication and centrifugation. Supernatants containing the *L. sakei* KBL_CE were filter-sterilized (pore size, 0.45 μm), lyophilized, and kept at -20°C until use.

The 3T3-L1 cell culture and differentiation assay

The 3T3-L1 preadipocyte cell line was obtained from the American Type Culture Collection (ATCC, USA) and cultured as described elsewhere (Hemati et al., 1997). Cells were cultured in 1% penicillin-streptomycin (PS)/DMEM containing 10% FBS (Gibco-BRL) [Lonza, Walkersville, MD, USA] at 37°C in a 5% CO_2 incubator. Differentiation was induced by incubating confluent cells for 6 days in differentiation medium (DM) containing 1% PS/DMEM, 10% FBS, 0.25 mM IBMX, 0.25 μM dexamethasone and 1 μg/ml insulin. The cells were then maintained in post-differentiation medium (DMEM containing 1% PS and 10% FBS), with replacement of the medium every 2 days. To examine the effects of the cell extract on the differentiation of preadipocytes to adipocytes, cells were cultured with MDI in the presence of various concentrations of cell extract (0 to 2.0 mg/ml).

Quantification of cell viability via MTT assay

The MTT assay is a standard colorimetric assay used to measure cellular proliferation (cell growth). Yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple formazan in the mitochondria of living cells. The number of surviving cells is directly proportional to the level of the formazan product created; the amount of colored product is directly proportional to the number of viable cells, and can be read on a multi-well scanning spectrophotometer (Rosen and Spegelman, 2006). The 3T3-L1 preadipocytes were treated with 1×10^5 *L. sakei* KBL_CE/well. After 48 h, the cells were incubated with MTT working solution [Promega] for 3 h. The fat was then removed from the plate, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. The absorbance of the resulting colored solution was quantified (absorbance 570 nm/reference 630 nm) using an ELISA plate reader [Bio-TEK Power-Wave XS, VT, USA]

Oil-red O staining

The cellular lipid content was assessed by Oil Red O staining. After the induction of differentiation, cells were washed twice with PBS, fixed in 3.7% formaldehyde (Sigma-Aldrich) in PBS for 1 h, and stained with Oil Red O [Cayman, USA] for 1 h. The stained fat droplets were dissolved in isopropanol containing 4% Nonidet P-40, and quantified by measuring the absorbance at 520 nm. Pictures were taken using an Olympus microscope.

$$\text{Lipid accumulation (\%)} = 100 - (A - B)/A \times 100$$

Where, A is the $A_{520\ nm}$ [control], B is the $A_{520\ nm}$ [LAB sample]

Statistical analysis

Statistical analyses were performed using Sigma Plot 10.0 [Systat software, USA]. Values are expressed as the means ± standard error (SE) from three independent experiments. Statistical significance was determined using the paired Student's *t* test.

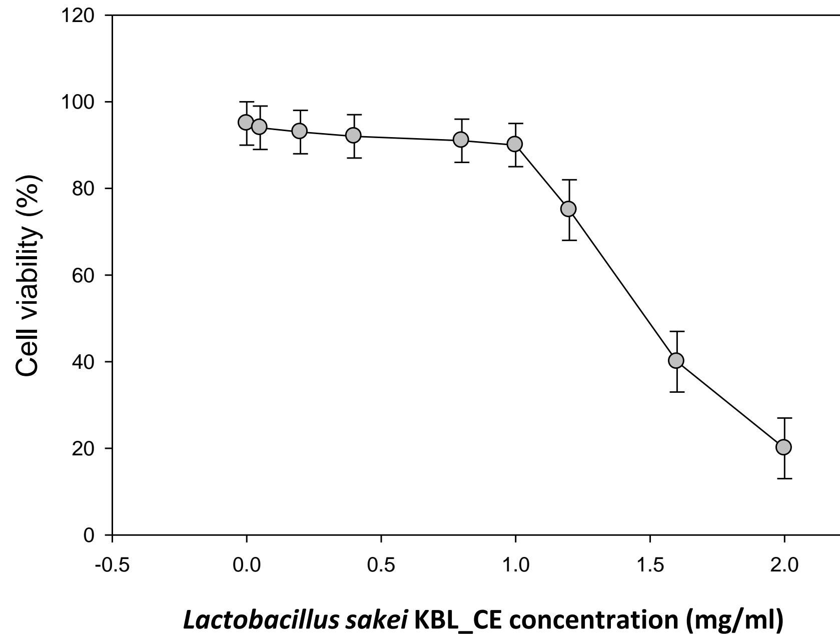

Figure 1. Effect of *Lactobacillus sakei* on the cell viability of post-confluence preadipocytes. The indicated concentrations of *L. sakei* KBL_CE were added to the differentiation medium at day 0. After 8 days of treatment, viability was determined by MTT assay. No cytotoxic effect was noted up to 1.0 mg/ml. Assays were performed in 3 replicates from 3 independent experiments. Values are means ± SEM ($p < 0.01$).

RESULTS

Effects of *L. sakei* KBL on cell viability in 3T3-L1 preadipocytes

To distinguish any inhibitory effect of *L. sakei* KBL_CE from a possible cytotoxic effect on 3T3-L1 preadipocytes, we treated cells with various concentrations of *L. sakei* KBL_CE and performed MTT assays. The extract failed to show any cytotoxic activity against 3T3-L1 cells up to 0.2 mg/ml (Figure 1).

Inhibition of adipocyte differentiation and lipid accumulation

To test whether *L. sakei* KBL_CE inhibited adipocyte differentiation, we used insulin, dexamethasone, and isobutylmethyl xanthine (differentiation medium, DM) to induce the differentiation of 3T3-L1 preadipocytes, treated the differentiating cells with [0.8×10^5 cells/well in a 6-well plate] *L. sakei* KBL_CE on day 0, and then changed the culture medium every 2 days for a total of 2 days. *L. sakei* KBL_CE were then switched to add to the 3T3-L1 cells at 2~8 days, and the adipocytes were stained with Oil Red O for visualization of fat droplets. The staining results showed that an 8-day treatment with various concentrations (0 to 1.0 mg/ml) of *L. sakei* KBL_CE during the differentiation period significantly and dose-dependently inhibited 3T3-L1 adipogenesis (Figure 2, lower panels) in terms of both cell differentiation (Figure 2) and lipid accumulation (Figure 3), compared with control cells. Among the tested concentrations of *L. sakei* KBL_CE, 1.0 mg/ml was the most effective at reducing the lipid content in differentiated cells (by 25% compared with control cells) (Figure 3). These results suggest that *L. sakei* KBL_CE inhibited the differentiation of 3T3-L1 preadipocytes by suppressing lipid accumulation.

DISCUSSION

The recent noticeable increases in the worldwide numbers of overweight and obese people are due in part to diet and lifestyle changes (Fuller, 1989). Some natural products have been shown to protect against obesity and have beneficial health effects, and have attracted the attention of researchers because of their relatively good safety profiles (Sato et al., 2008). Among these natural products, probiotic preparations comprising dead cells or their metabolites have been shown to exert biological responses (Ma et al., 2008; Chang et al., 2010). Among the various beneficial health effects of probiotics, their biological impact on obesity has generated considerable interest. For example, the probiotic species, *L. plantarum*,

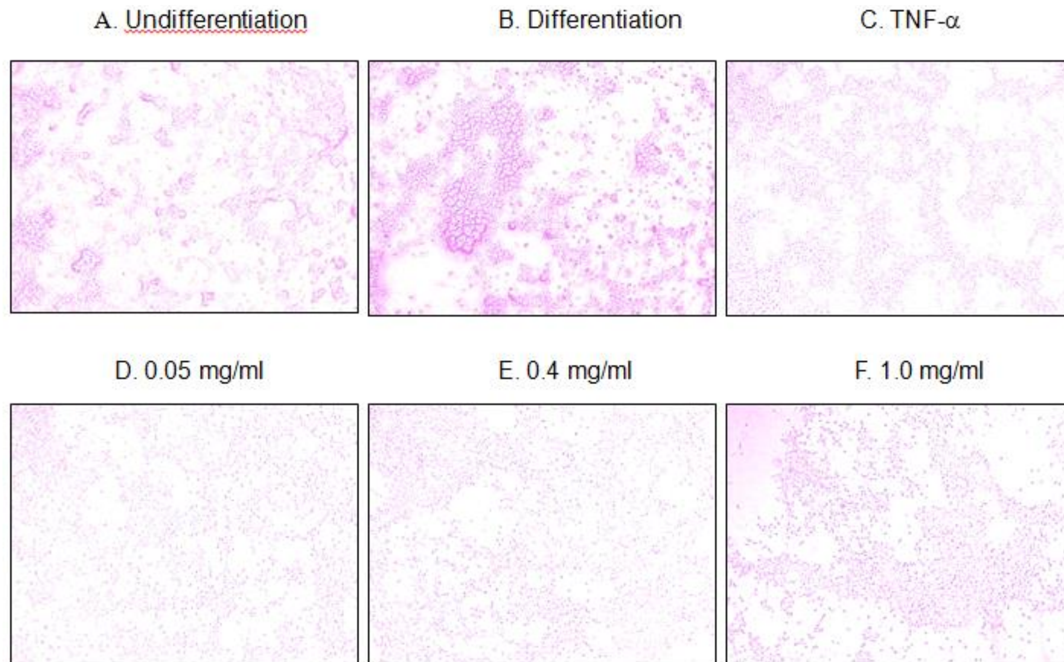

Figure 2. Morphological examination of undifferentiated cells (A), control differentiated cells (B), tumor-necrosis factor (TNF)-α treated control cells (C), cells treated with various concentrations of *L. sakei* KBL_CE (D, 0.05 mg/ml; E, 0.4 mg/ml; F, 1.0 mg/ml). Confluent 3T3-L1 cells were treated with various concentrations of *L. sakei* KBL_CE for 8 days and lipid accumulation was measured by Oil Red O staining. A significant inhibitory effect [using red staining as a proxy for differentiation here and counting the red-stained cells] was noticed.

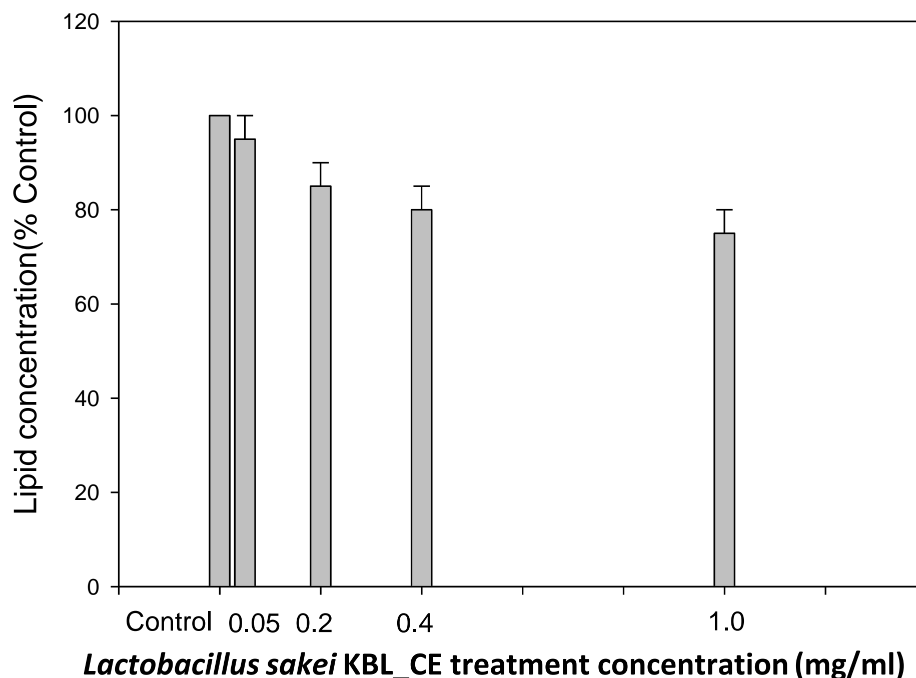

Figure 3. Inhibitory effect of *L. sakei* KBL_CE on lipid accumulation in 3T3-L1 adipocytes. Confluent 3T3-L1 cells were treated with various concentrations of *L. sakei* KBL_CE for 8 days, and lipid accumulation was examined by Oil Red O staining. Assays were performed in three replicates from three independent experiments. Values are means ± SEM (p<0.01).

was shown to have specific biological effects (including anti-obesity effects) *in vitro* (Cho et al., 2006). In this study, the potential anti-obesity effects of *L. sakei* KBL_CE were investigated through cell viability assays and Oil red O staining of 3T3-L1 cells treated with various concentrations of the extract either in maintenance culture or during differentiation. For increased adipose tissue to be created, the number or size of adipocytes must increase by either proliferation or differentiation (Ambati et al., 2007). Conversely, the reduction of adipose tissue mass involves the loss of lipids through lipolysis, the inhibition of preadipocyte proliferation, and/or the decreased differentiation of mature adipocytes. Treatment of 3T3-L1 cells with various concentrations (0 to 1.0 mg/ml) of *L. sakei* KBL_CE did not significantly alter cell viability, indicating that the inhibitory effect of *L. sakei* KBL_CE on lipid accumulation was due not to reduced cell viability. Such reduction was seen up to a 2.0 mg/ml. Instead, we observed decreased adipogenesis in *L. sakei* KBL _CE-treated cells compared to control cells (Figure 2), indicating that the extract reduced the lipid accumulation in mature adipocytes. The loss of fat mass can be partly attributed to lipolysis, in which triglycerides are broken down into glycerol and fatty acids in adipocytes. Together, our results indicate that *L. sakei* KBL_CE may contain components that inhibit lipid accumulation in 3T3-L1 cells. The observed inhibitory effects were more significant in cells treated with a higher concentration of *L. sakei* KBL_CE versus a lower concentration of *L. sakei* KBL_CE (Figure 3). Thus, our results confirm the potential anti-obesity effects of *L. sakei* KBL_CE and suggest that *L. sakei* KBL may have similar effects.

It is not clear what compounds of *L. sakei* KBL worked as the main principles of the anti-obesity effects. Therefore, *L. sakei* KBL can be used as a useful material not only for the production of fermented foods such as kimchi, but also for the investigation of unknown compounds for the anti-obesity effects. Further studies may provide additional insights into the active compounds of *L. sakei* KBL that have specific effects on obesity.

Conclusion

L. sakei KBL_CE exerted inhibitory effects on adipocyte differentiation and lipid accumulation, suggesting that *L. sakei* KBL might have further implication for *in vivo* antiobesity effect and could thus possibly be developed as a therapeutic substance for preventing or treating obesity.

Conflict of interests

The authors did not declare any conflict of interest.

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