academicJournals

Vol. 13(26), pp. 2626-2636, 25 June, 2014
DOI: 10.5897/AJB2014.13772
Article Number: C2DF31645607
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Chemical analyses, antibacterial activity and genetic diversity assessment of some Egyptian *Citrus* spp. cultivars

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Received 4 March, 2014; Accepted 9 June, 2014

Citrus species are among the most important fruit trees in the world and are considered as a major export product of Egypt. Forty-eight *Citrus* L. accessions representing six citrus groups (orange, mandarin, lemon, sour orange, grape fruit and pummelo) were collected. Chemical proprieties including pH, total acidity, total soluble solids and ascorbic acid of different fruit juices were determined. Eight accessions representing different citrus groups were screened for their antibacterial activity against five pathogenic bacteria (*Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Micrococcus* spp. and *Bacillus pumilus*). Lemon and lime accessions exhibited the highest antibacterial activity compared to the standard antibiotics (ampicillin and streptomycin). However, grapefruit and pummelo accessions showed no inhibitory effect. Inter-simple sequence repeats (ISSR) markers were used to study the genetic diversity and phylogenetic relationships among citrus accessions. The highest level of polymorphism (71%) was detected amongst lemon and lime accessions, whereas, the lowest percentage of polymorphism (18%) was identified within the sour orange group. The phylogenetic tree separated the varieties into discrete clusters according to their respective citrus group. Citrus groups were initially divided into two main clusters at 0.18 level of similarity. Lemon, lime, mandarin and sour orange were grouped in the first cluster, while sweet orange, grapefruit and pummelo were nested in the second cluster.

Key words: Citrus, genetic diversity, ISSR markers, chemical analyses, antibacterial.

INTRODUCTION

The genus *Citrus* L. (family *Rutaceae*; sub-family *Aurantioideae*) includes some of the principal fruit crops of worldwide importance such as the citrons (*C. medica* L.), lemons [*C. limon* (L.) Osbeck], limes [*C. aurantifolia* (Christm.) Swingle], mandarins (*C. reticulate* Blanco), sour oranges (*C. aurantium* L.), sweet oranges [*C. sinensis* (L.) Osbeck], grapefruits (*C. paradisi* Macf.) and pummelos [*C. maxima* (Burm.) Merr.] (Golein et al., 2012). Citrus fruits are recognized as an important component of the human diet, providing a variety of constituents important to human nutrition, including vitamin C (ascorbic acid), folic acid, potassium, flavonoids, coumarins, pectin and dietary fibers (Dugo and Di Giacomo, 2002). Flavonoids in citrus have a broad spectrum of

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biological activities including antibacterial, antioxidant, antidiabetic, anticancer, analgesic, anti-inflammatory and antianxiety (Sidana et al., 2013).

Worldwide production of citrus reached more than 129 million tons from cultivated trees in 140 countries around the world FAOSTAT (2012). The statistics Division of the Food and Agriculture Organization of the \United Nations (FAO) http://faostat.fao.org/, making citrus the leading cultivated tree crop. Citrus production in the Mediterranean Basin (22,441 thousand tons) is third only to China and Brazil (FAO, 2012) and accounts for about 20% of the world citrus production and about 60% of the world fresh citrus trade (CLAM, 2007). Spain is the leading producing country, whereas Italy and Egypt rank second and third, respectively. Egypt represents about 15% of the total citrus production in the Mediterranean Basin (CLAM, 2007) and is considered the ninth largest citrus producer in the world (Ahmed, 2012) with a global market share of 3.1% of the world citrus production FAOSTAT (2012) The statistics Division of the food and Agriculture Organization of the United Nations (FAO) http://faostat.fao.org/. The production is mainly composed of oranges, mandarins and limes, which represent more than 98.8% of the total citrus area (Eid and Guindy, 2008).

Evaluation of genetic diversity and genetic relationships among various accessions is of fundamental importance for plant breeding programs. This information can provide predictive estimates of genetic variation within a species, thus facilitating breeding material selection (Qi et al., 2008). In recent years, the progress made in the development of DNA based marker systems has advanced our understanding of genetic resources diversity and their gene mapping (Kalia et al., 2011). Many citrus genetic maps have been developed over the past decade (Chen et al., 2007; Roose, 2007); each genetic map has a different mapping population type and size, genome coverage, and marker systems. Most of these maps were covered by a majority of randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeats (ISSR), and simple sequence repeats (SSR) markers (Gulsen et al., 2010). Among these markers, ISSR has been widely used to assess the genetic diversity between different citrus species (Sankar and Moore, 2001; Uzun et al., 2010; Yang et al., 2010).

The objectives of this study were to analyze the chemical properties of forty-eight citrus accessions, explore their genetic diversity at intra-and interspecific levels of variation and screen the antibacterial activity of a representative sample from each citrus group against human pathogenic bacteria.

MATERIALS AND METHODS

Plant materials

Samples from young leaves and fresh fruits were collected from 48 individual trees, representing six citrus groups (sweet orange, mandarin, lemon, lime, sour orange, grape fruit and pummelo) from Moshtohor on-farm collection, Faculty of Agriculture, Benha University (Table 1)

Preparation of juice

At the time of maturation and ripeness, ten fresh fruits for each accession were sampled. Fruits were washed in running tap water in the laboratory, surface sterilized with 70% alcohol, rinsed with sterile distilled water and cut open with a sterile knife and the juice pressed out into a sterile universal container. Then juice was filtered using 0.45 membrane filter (Millipore©, USA) into another sterile container to remove the seeds and other tissues.

Determination of pH and total acidity

Total acidity of the juices was determined by titration method as reported by Rekha et al., (2012). Fruit juice was diluted to 10% with distilled water and then titrated against 0.1N NaOH (standardized using standard Oxalic acid) using Phenolphthalein indicator. The end point was noted when the color changed from colorless to pale pink. All measures were done in triplicate and dilution factor was considered; total acidity was calculated in terms of citric acid using the following formula, Acidity (g/100 mL) = Normality of the juice x Equivalent weight of citric acid. The pH of citrus juice was determined using a digital pH meter (Thermo©, USA).

Determination of total soluble solids (TSS)

Total soluble solids (TSS) were measured using digital refractometer (Atago Co., Ltd., Tokyo, Japan). All measures were done in triplicate; the TSS results were reported as ° Brix.

Estimation of ascorbic acid (vitamin C) content

Ascorbic acid content in fruit juice was determined by the 2, 6 dichlorophenol-indophenol titrimetric method according to AOAC method No. 967.21 (AOAC, 2000). All measures were done in triplicate; the vitamin C content was expressed as mg/100 ml.

Bacterial strains and cultural conditions

The antibacterial tests were carried out against five human pathogenic bacteria; *E. coli*, *S. typhi*, *S. aureus*, *Micrococcus* spp., *B. pumilus*. The bacterial cultures were supplied from Microbiology Department, National Organization of Drug Control and Research, Giza, Egypt. The cultures were maintained on nutrient agar slants and sub-cultured for 24 h before use.

Antibacterial activity test

The agar diffusion method (CLSI, 2002; Prescott et al., 2002) was used as a preliminary assay for testing the antibacterial effect of crude juice extracts of eight citrus accessions (Valencia orange, Balady mandarin, Eureka and Rough lemon, Balady lime, Balady sour orange, Duncan grapefruit and Egyptian pummelo) that represent different citrus groups. A previously liquefied and sterilized nutrient agar medium (20 ml) was poured into Petri-plates of 100 mm size (to make uniform thickness) and kept for solidifying. One milliliter of 10^9 a log phase bacterial culture was spread over the solidified media. Wells of 10 mm diameter were made in each

Table 1. List of citrus accessions used in this study.

No.	NGB* accession	Common name	Scientific name	No	NGB* accession	Common name	Scientific name
Sweet orange				**Lemon and Lime**			
1	127	San Gwen	*C. sinensis*	27	153	Eureka	*C. limon*
2	128	Balady	*C. sinensis*	28	154	Eureka	*C. limon*
3	129	Jaffa	*C. sinensis*	29	155	Variegated Pink	*C. limon*
4	130	Regular bearing	*C. sinensis*	30	156	Variegated Pink	*C. limon*
5	131	Succari	*C. sinensis*	31	157	Sweet lemon	*C. limetta*
6	132	Mouzambique	*C. sinensis*	32	158	Sweet lime	*C. limetta*
7	133	Blood Balady	*C. sinensis*	33	159	Sweet lime	*C. limetta*
8	134	Tunisi	*C. sinensis*	34	160	Succari lime	*C. limetta*
9	135	Navel	*C. sinensis*	35	161	Rough lemon	*C. jambhiri*
10	136	Khalili White	*C. sinensis*	36	162	Balady lime	*C. aurantifolia*
11	137	Khalili Red	*C. sinensis*	**Sour orange**			
12	138	Greek compressed	*C. sinensis*	37	163	Balady	*C. aurantium*
13	139	Tanneriffe	*C. sinensis*	38	164	Balady	*C. aurantium*
14	140	Centrial	*C. sinensis*	39	165	Barzi	*C. aurantium*
15	141	Mezazie	*C. sinensis*	**Grapefruit**			
16	142	Mafred	*C. sinensis*	40	166	Grapefruit	*C. paradisi*
17	143	Roja	*C. sinensis*	41	167	White Grapefruit	*C. paradisi*
18	144	Valencia	*C. sinensis*	42	168	Duncan	*C. paradisi*
19	145	Golden Nagen	*C. sinensis*	43	169	Marsh	*C. paradisi*
20	146	Hamlin	*C. sinensis*	**Pummelo**			
Mandarin				44	170	Broad leaf	*C. maxima*
21	147	Cleopatra	*C. reshni*	45	171	Egyptian	*C. maxima*
22	148	Clementine	*C. reticulata*	46	172	Rabehe	*C. maxima*
23	149	Clementine	*C. reticulata*	47	173	Gizawe	*C. maxima*
24	150	Balady	*C. deliciosa*	48	174	Moneybi	*C. maxima*
25	151	Satsuma	*C. unshiu*				
26	152	Santara	*Citrus spp*				

*NGB: National gene Bank, Giza, Egypt.

plate with a sterilized stainless steel borer. One hundred µl of fresh juice sample was poured into the well and compared to 100 µl Ampicillin and Streptomycin antibiotics (100 µg/ml) (Serva©, Germany) as standards. Sterilized H_2o was used as a negative control. The plates were performed in triplicates and then left standing for 3 hrs at 4°C (Sultana et al., 2012) for proper diffusion of the tested juices. After diffusion process, all the Petri plates were incubated at 37°C for 24 h, and then they were observed for zones of inhibition.

DNA extraction and ISSR analysis

Total DNA extraction was isolated using DNeasy Plant Mini Kit (Qiagen©, Germany) according to the manual procedures. Out of 30 tested ISSR primers, a total of 13 primers (Table 2), that generated clear reproducible banding patterns, were chosen for the final analysis. PCR reaction was performed in 25 µl reaction mix containing 1 X PCR buffer, 2 mM $MgC1_2$, 0.2 mM of each dNTPs, 1 µM oligonucleotide primer, 25 ng genomic DNA and 1 unit of Taq DNA polymerase (Promega©, USA). Amplification was performed in a 96-well Thermal Cycler (BioRad©, USA) under the following conditions: 3 min at 94°C for 1 cycle, followed by 1 min at 94°C, 1 min at annealing temperature (Table 2) , and 2 min at 72°C for 35 cycles, and 7 min at 72°C for a final extension step. PCR products were separated by electrophoresis on a 1.5% agarose gel stained by ethidium bromide and photographed by gel documentation (BioRad©, USA). The banding patterns generated by ISSR markers were analyzed and compared to determine the genetic diversity and relatedness among different citrus genotypes. The amplified fragments were scored either as present (1) or absent (0). The genetic similarity and similarity matrix among genotypes were estimated according to Dice coefficient (Sneath and Sokal, 1973) and based on Nei's (1972) genetic distance. Dendrograms showing phylogenetic relationships were constructed using the Un-weighted Pair Group Method with Arithmetic Averages (UPGMA) by Phoretix 1D software (TotalLab, UK).

Statistical analysis

The physiochemical data were statistically analyzed using SPSS Software for Windows (version 21; SPSS Inc., USA) to evaluate the significant differences at $p<0.05$ and to construct the correlation matrix. Antibacterial activity data were analyzed using the MSTATC analysis software according to Snedecor and Cochran (1980).

Table 2. List of primer names, sequences, annealing temperatures, total number, and size of amplicons and number of polymorphic bands as revealed by ISSR markers among the 48 citrus accession.

Primer	Sequence	Annealing temperature (°C)	PCR amplified fragments		Number of polymorphic bands
			Number	size range (bp)	
H_{12}	$(GA)_8YT$	41.0	19	140-1300	19
H_{13}	$(GA)_8YC$	42.5	22	90-2434	22
H_{14}	$(GA)_8YG$	44.0	20	180-1160	20
H_{15}	$AG)_8YT$	52.0	21	180-1510	20
H_{16}	$(AG)_8YC$	56.5	26	100-2530	26
H_{17}	$(AG)_8YG$	59.5	24	185-1466	24
H_{21}	$(GT)_8YC$	60.5	22	180-3470	22
H_{29}	$(GACA)_4AT$	41.5	19	195-1700	19
P_2	$(CA)_6GG$	48.0	25	180-1500	25
P_3	$(CA)_6AC$	42.5	21	195-1425	21
P_4	$(GTG)_3GC$	52.5	23	180-1360	23
P_{11}	$(GAG)_3GC$	45.0	17	220-1280	17
P_{16}	$ACG(GT)_7$	50.0	19	190-810	18
Total	-	-	278	-	275
Average	-	-	21.4	-	21.2

RESULTS

Chemical analysis of citrus fruit juices

Some chemical properties including pH, total acidity, total soluble solids and ascorbic acid of fresh fruit juices are shown in Table 3. The results show that, pH ranged from 2.50 in variegated pink lemon (Accessions NGB 155 and NGB-156) to 6.79 in Tunisi sweet orange fruits (NGB-134). However, titratable acidity ranged from 0.40% in Sweet lemon (NGB-157) fruits to 8.82 g citrate/100 ml in Balady sour orange (NGB-164). The highest solid soluble content (12.2^{o}Brix) was determined in Cleopatra mandarin (NGB-147), while the lowest soluble solids content (5.9°Brix) was detected in Variegated pink lemon (NGB-155 and NGB-156). The ascorbic acid content ranged from 4.2 mg/100 ml in Sweet lemon (NGB-157) to 56.9 mg/100 ml in Hamlin sweet orange (NGB-146) and Egyptian pummelo (NGB-171).

Antibacterial screening of citrus juice

The antibacterial activity of fresh juice of eight accessions representing different citrus groups compared to standard antibiotics (Ampicillin and Streptomycin) was screened against five pathogenic bacteria using agar diffusion assay (Table 4). Lemon and lime accessions exhibited the highest antibacterial activity against tested pathogenic bacteria, followed by Balady mandarin (NGB-150) which showed a moderate activity. Whereas, Valencia sweet orange (NGB-144) and Balady sour orange (NGB 163) displayed a feeble bioactivity. On the other hand, Duncan grapefruit and Egyptian pummelo accessions had no inhibitory effect. In general, Balady lime (NGB-162) had the highest antibacterial activity compared to other tested *Citrus* species (Table 4 and Figure 1).

Genetic diversity within *Citrus* genus

The genetic diversity of 48 citrus accessions was studied using 13 selected ISSR primers (Table 2) which generated reproducible and scorable patterns, compared to the other primers, which produced smears, or fuzzy patterns that could not be scored. Out of the 278 total amplified amplicons, 275 were polymorphic. The number of fragments amplified per primer varied from 17 (primer P_{11}) to 26 (Primer H_{16}), with an average of 21.4 fragments per primer (Table 2).

The highest number of amplified polymorphic amplicons was identified among lemon and lime group, which resulted in 71% polymorphism (Table S1). However, the lowest number of amplified polymorphic amplicons was detected among sour orange group, which resulted in 18% polymorphism (Table S1).

Genetic differentiation between species

The analysis of amplified amplicons produced by ISSR primers revealed the presence of positive unique markers that allowed the differentiation between different citrus species (Table S2). Six *Citrus* species (*C. sinensis*, *Citrus* spp., *C. limetta*, *C. jambhiri, C. aurantifolia* and *C. aurantium*) were discriminated by species specific unique ISSR markers (Table S2). The number of unique markers ranged from one in *C. limetta* species to 33 in *C. aurantium* species.

Table 3. Total soluble solids (TSS), pH, acidity percent and ascorbic acid of citrus fruits juice*.

Variety	Citrus Acces.	TSS Brix°		pH		Acidity gm citrate/100 ml		Ascorbic acid**	
	127	ND		ND		ND		ND	
	128	8.7	± 0.12	3.97	± 0.02	2.00	± 0.07	35.0	± 1.6
	129	9.1	± 0.10	4.09	± 0.00	1.84	± 0.07	35.3	± 0.46
	130	8.8	± 0.06	3.97	± 0.00	1.52	± 0.07	43.7	± 0.46
	131	11.0	± 0.00	6.28	± 0.00	0.76	± 0.07	21.1	± 0.46
	132	9.6	± 0.06	3.77	± 0.02	2.56	± 0.07	53.7	± 2.09
	133	9.7	± 0.06	3.94	±0.04	2.16	± 0.12	37.4	± 1.82
	134	11.2	± 0.20	6.79	± 0.00	0.76	± 0.07	28.4	± 0.00
	135	11.6	± 0.06	4.36	± 0.01	1.16	± 0.07	38.9	± 0.91
Sweet orange	136	10.7	± 0.11	3.99	± 0.03	2.12	± 0.07	33.4	± 0.46
	137	9.2	± 0.00	3.97	± 0.00	1.52	± 0.14	41.6	± 0.91
	138	8.8	± 0.00	4.16	± 0.02	1.56	± 0.00	42.9	± 1.20
	139	12.1	±0.06	4.08	± 0.00	1.20	± 0.00	26.3	± 1.20
	140	10.2	± 0.06	4.20	± 0.01	1.96	± 0.07	37.7	± 1.20
	141	11.7	± 0.12	4.17	± 0.01	1.40	± 0.07	27.1	± 1.20
	142	9.8	± 0.06	3.99	± 0.02	2.64	± 0.12	41.3	± 1.64
	143	11.3	± 0.12	3.75	± 0.04	2.40	± 0.00	45.0	± 0.00
	144	10.3	± 0.12	3.53	± 0.03	3.85	± 0.12	38.2	± 0.91
	145	10.4	± 0.06	3.87	± 0.01	2.24	± 0.18	37.4	± 0.46
	146	10.8	± 0.00	4.11	± 0.00	1.24	± 0.07	56.9	±0.00
	147	12.2	± 0.06	3.70	± 0.00	0.78	± 0.01	16.8	± 0.00
	148	10.7	± 0.00	3.77	± 0.07	0.75	± 0.00	23.5	± 0.01
Mandarin	149								
	150	10.0	± 0.06	3.41	± 0.02	2.08	± 0.07	4.3	± 0.40
	151	9.1	± 0.00	3.59	± 0.13	0.68	± 0.00	14.2	± 0.06
	152	11.5	± 0.7	3.20	± 0.17	1.22	± 0.00	29.3	± 0.06
	153	8.1	± 0.00	2.82	± 0.01	6.46	± 0.07	22.9	± 0.79
	154								
	155	5.9	± 0.06	2.50	± 0.00	4.90	± 0.07	22.7	± 0.00
	156								
Lemon and lime	157	7.1	± 0.12	5.94	± 0.01	0.40	± 0.07	4.2	± 0.46
	158	10.1	± 0.06	5.91	± 0.04	0.84	± 0.12	5.5	± 0.79
	159								
	160	ND	ND	ND	ND				
	161	11.0	± 0.06	2.72	± 0.03	6.55	± 0.10	22.1	± 0.00
	162	12.0	± 0.00	2.68	± 0.00	5.17	± 0.00	26.3	± 0.46
	163	10.9	± 0.06	2.97	± 0.01	7.26	± 0.07	42.7	± 2.09
Sour orange	164	10.1	± 0.06	3.20	± 0.01	8.82	± 0.07	29.5	± 1.82
	165	9.0	± 0.00	2.98	± 0.00	7.70	± 0.12	21.3	± 0.79
	166	ND		ND		ND		ND	
Grapefruit	167	ND		ND		ND		ND	
	168	8.7	± 0.06	3.41	± 0.00	3.29	± 0.07	39.8	± 0.91
	169	9.8	± 0.06	3.37	± 0.02	3.13	± 0.00	43.2	± 1.64
	170	11.0	± 0.06	3.28	± 0.03	4.29	± 0.07	54.2	± 0.46
	171	11.0	± 0.06	3.39	± 0.02	4.25	± 0.07	56.9	± 1.37
Pummelo	172	9.9	± 0.06	3.52	± 0.00	1.56	± 0.05	19.2	± 0.00
	173	9.9	± 0.06	3.55	± 0.00	1.37	± 0.05	22.2	± 0.00
	174	9.0	± 0.00	2.85	± 0.00	4.83	± 0.18	27.8	± 0.01

ND, Not Determined; *Means are followed by standard deviation, ** Ascorbic acid was determined as mg/ 100 ml.

Table 4. Antibacterial activity of fresh juice of some citrus accessions compared to standard antibiotics (Ampicillin and Streptomycin) against tested pathogenic bacteria.

Tested citrus juice/ antibiotic	Variety	Zone of Inhibition (mm)*									
		E. coli		*S. typhi*		*S. aureus*		*Micrococcus* spp.		*B. pumilus*	
Valencia (144)	Sweet orange	2	± 0.58	3	± 0.58	4	± 0.58	3	± 0.58	2	± 0.58
Balady (150)	Mandarin	16	± 0.10	18	± 0.58	16	± 0.58	21	± 0.58	18	± 0.58
Eureka (NGB 153)	Lemon	18	± 0.58	24	± 1.00	28	± 1.53	26	± 1.53	20	± 1.53
Rough (NGB 161)	Lemon	18	± 1.15	22	± 1.00	25	± 1.73	29	± 3.2	23	± 3.51
Balady (NGB 162)	Lime	30	± 1.53	29	± 3.00	25	± 1.00	26	± 1.53	25	± 1.00
Balady (NGB 163)	sour orange	3	± 0.58	4	± 0.58	3	± 0.58	5	± 0.58	4	± 0.58
Duncan (NGB 168)	Grapefruit	-	-	-	-	-	-	-	-	-	-
Egyptian (NGB 171)	Pummelo	-	-	-	-	-	-	-	-	-	-
Ampicillin (100 µg/ ml)		25	± 1.15	26	± 0.58	20	± 1.53	31	± 1.00	25	± 1.73
Streptomycin (100 µg/ ml)		20	± 1.00	15	± 1.00	19	± 0.58	22	± 0.58	20	± 3.00

*Means followed by the Standard Deviation (SD)

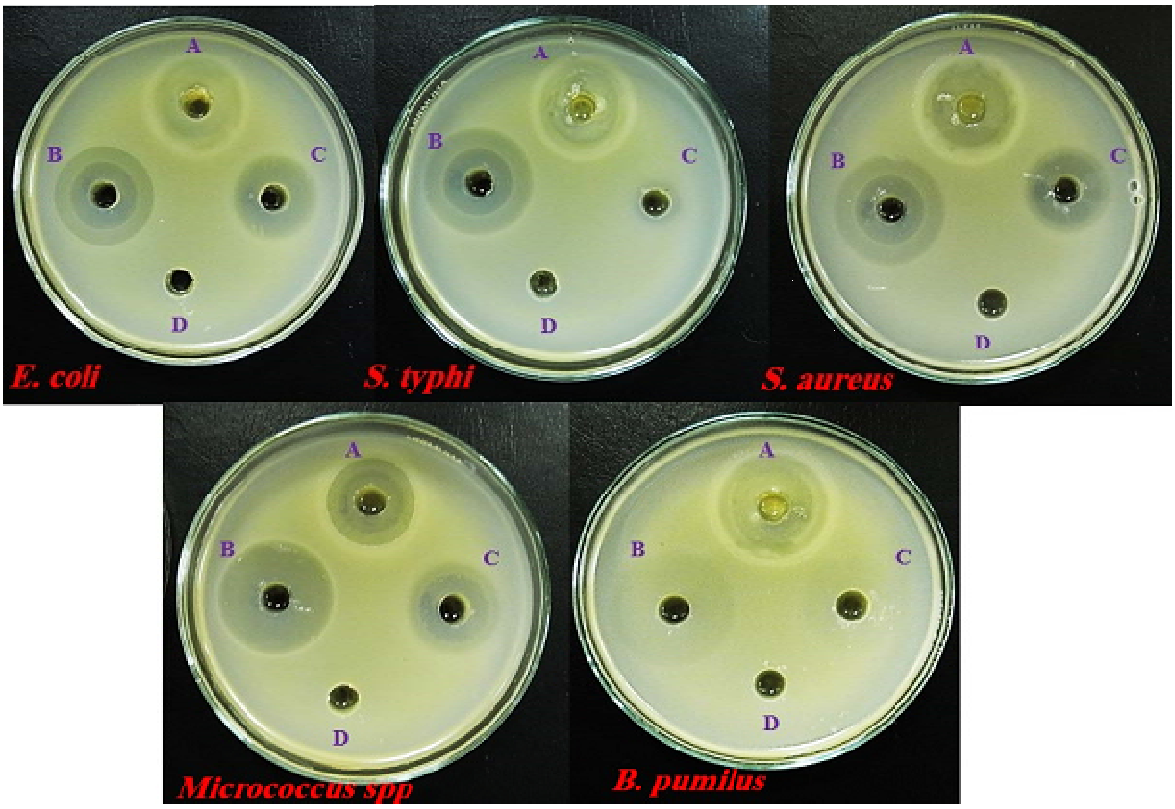

Figure 1. Antibiograms of Balady lemon juice (NGB-162) and used antibiotics (100 µg/ml) against tested bacteria. A, Balady lemon juice (NGB-162) juice; B, Ampicillin; C, Streptomycin; D, Sterile water.

Cluster analysis

Similarity matrix based on the ISSR data was calculated according to Dice coefficient (Sneath and Sokal, 1973). The highest genetic similarity (0.98) was identified among the accessions belonging to sweet orange group. However, the highest genetic diversity (0.12 similarity coefficient) was detected between lemon and lime group and sweet orange group. The UPGMA dendrogram based on the 278 ISSR amplified bands of the 48 accessions

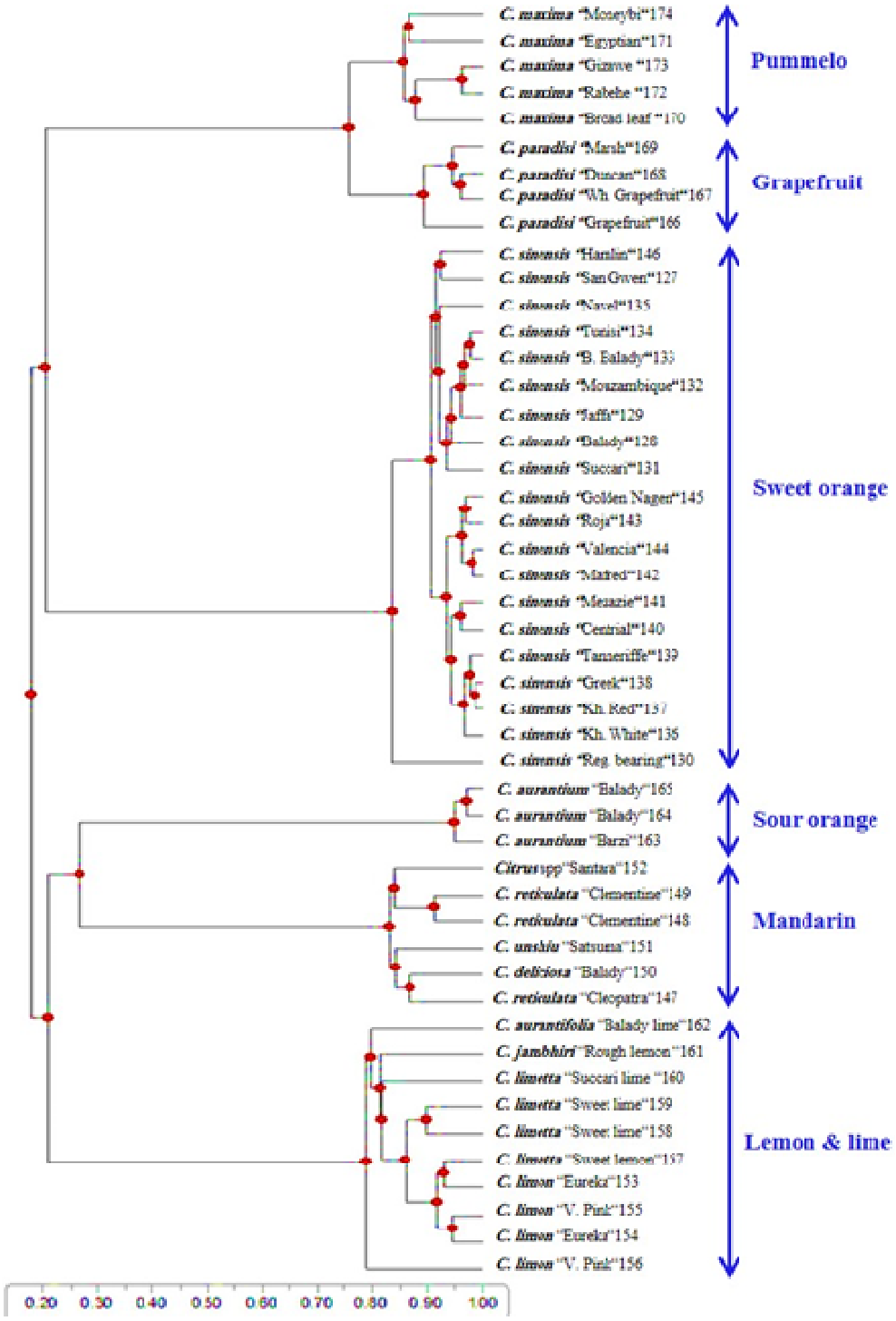

Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance for the forty-eight citrus accessions constructed by cluster analysis of ISSR markers. The numbers following the *Citrus* species are the accessions numbers.

(Figure 2), showed six well-defined lineages, corresponding to the different citrus groups (lemon, mandarin, sour orange, sweet orange, grapefruit and pummelo). The phylogenetic tree (Figure 2) divided citrus genotypes into two main clusters at 0.18 level of similarity; the first cluster included three sub-lineages; lemon, mandarin and sour orange groups, while the second cluster comprised sweet orange, grapefruit and pummelo groups.

DISCUSSION

Chemical properties of citrus fruits juice

Chemical composition of genetic resources is an essential identification process in monitoring of the genetic quality during improvement and conservation (IPGRI, 1999). Citrus is a good source of vitamin C, which is the most important nutrient component in citrus fruit juice (Xu et al., 2008). Our study showed that sweet oranges and pummelo fruits are good sources of vitamin C. These findings are compatible with other results published by other workers (Pichaiyongvongdee and Haruenkot, 2009; Hashempour et al., 2013). On the other hand, lemon and lime varieties showed a moderate ascorbic acid content (4-26 g/100 ml), which is in agreement with results reported by Rekha et al. (2012).

Antibacterial activity

Citrus varieties are considered a rich source of secondary metabolites which have the ability to produce a broad spectrum of biological activities (Johann et al., 2007; Ghasemi et al., 2009). Results of the current study showed a promising antibacterial activity of selected citrus fruit juice against some human pathogenic bacteria (*E. coli*, *S. typhi*, *S. aureus*, *Micrococcus* spp. and *B. pumilus*). Among the tested accessions (Valencia orange, Balady mandarin, Eureka and Rough lemon, Balady lime, Balady sour orange, Duncan grapefruit and Egyptian pummelo), that represent different citrus groups, lime and lemon juices exhibited the highest biological activity. Lemons and limes have been known as an important medicinal plants and the potentiality of their juices as antimicrobial agents was previously confirmed (Tomotake et al., 2006; Jayana et al., 2010; Bansode and Chavan, 2012; Hindi and Chabuck, 2013).

In general, antimicrobial activity of Citrus may be referred to their rich content of flavonoids. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Burt, 2004). Lemon juice is characterized by the presence of significant amounts of the flavones, flavanones, hesperidin and eriocitrin (Gattuso et al., 2007). The bioactivity of lemon juice containing-flavonoids like luteolin and apigenin (Gattuso et al., 2007) have been previously reported (Cushnie and Lamb 2005).

Genetic analysis of the *Citrus* genus

Understanding the genetic variation within and among populations is essential for the establishment of effective and efficient conservation practices for plant genetic resources (Yang et al., 2010). ISSR markers technique has been known as a rapid, reproducible and useful method for distinguishing among different cultivars and clustering genotypes in the citrus species (Siragusa et al., 2006; Yang et al., 2010). In the present study, 13 primers produced clear, species-specific fingerprint patterns with all samples and were sufficient to discriminate varietal groups of citrus and produced results consistent with previous studies (Yang et al., 2010). Polymorphism analysis exhibited an average of 99.2% polymorphism among the forty-eight accessions under study. Similarly, Hussein et al., (2003) reported an average of 82.4% polymorphism generated by eight ISSR primers among fourteen Egyptian citrus genotypes. The UPGMA phylogenetic tree (Figure 2) clearly splits the Egyptian accessions into two main clusters. The first cluster included lemon, lime, mandarin and sour orange varieties. This finding is compatible with the hypothesis that, lemon originated from citron and sour orange, with sour orange being the maternal parent (Nicolosi et al., 2000; Gulsen and Roose, 2001). Also, clustering of mandarins and sour oranges into two sub-clusters, which is in agreement with the suggestion that mandarin, is the paternal parent of sour orange (Li et al., 2010). On the other hand, the second cluster included sweet orange, grapefruit and pummelo accessions. This grouping is in accordance with the hypothesis that pummelo is the maternal parent of sweet oranges (Nicolosi et al., 2000; Froelicher et al., 2011). Our study showed that, grapefruits were much closer to pummelos (0.76 genetic similarity) than sweet oranges (0.20 genetic similarity), which could confirm that grapefruit was derived from a backcross with pummelo (Barkley et al., 2006; Pang et al., 2007). In conclusion, the phylogenetic tree based on ISSR markers, separated the citrus varieties into discrete clusters according to their respective citrus group.

Intra- variation within each citrus group

Lemons and Limes

Lemons and limes account for 10.3 and 0.21% of the total citrus cultivated area in Egypt, respectively (Eid and Guindy, 2008). All the accessions were grouped in one lineage; however, the variegated pink lemon accession (NGB-156) was located separately from the other varieties exhibiting a 0.79 genetic similarity. The high genetic similarity found amongst lemons and limes was previously reported by other workers (Federici et al., 1998; Nicolosi et al., 2000). This genetic overlapping could be referred to the suggestion that, (*C. medica*) an ancestral of citrus species gave rise to lemons, limes, and rough lemons through various hybridization events (Barkley et al., 2006).

Mandarins

Mandarins account for 26.4% of the total citrus area in

Egypt. In the present study, a high degree of genetic similarity ranging from 0.83 to 0.90 was detected between the six mandarin accessions, although they belonged to four different species (*C*. *reticulate*, *C*. *delciosa*, *C*. *unshiu* and *Citrus* spp.). The phylogenetic dendrogram showed that, mandarins were closer to sour oranges than lemon and lime cultivars, which is in agreement with other results published by EL-Mouei et al., (2011).

Sour oranges

Sour orange is the most widely used citrus root stocks in Egypt (Eid and Guindy, 2008) and worldwide (Siraguse et al., 2006). A high genetic similarity coefficient ranging from 0.95 to 0.97 was detected among the three sour orange accessions. Similarly, Hussein et al., (2003) reported a high genetic similarity among Spanish, Balady and Brazilian sour orange accessions belonging to *C. aurantium* species.

Sweet oranges

Orange production accounts for about 61% of total citrus production in Egypt (Eid and Guindy, 2008). Three principal varieties of oranges are produced in Egypt; Navel (35%), Valencia (18.4%) and Baladi (7.0%). ISSR markers revealed a high level of genetic similarity ranging from 0.83 to 0.98 among the twenty orange accessions. This narrow genetic base among the sweet orange accessions has been previously reported in many publications (Fang and Roose, 1997; Targon et al., 2000; Snoussi et al., 2012).

Grapefruits and Pummelos

Grapefruit is the fourth economically most important citrus fruit in the world (Uzun et al., 2010). A high level of genetic similarity was detected among grapefruit accessions ranging from 0.89 to 0.97; this narrow genetic base among the grapefruit cultivars has been reported in previous publications using different molecular markers (Fang and Roose, 1997; Corazza-Nunes et al., 2002). The high level of similarity within the grapefruit group supported the hypothesis that the majority of grapefruit cultivars were derived from the same ancestral tree by mutations (Gmitter, 1995). Pummelo has played an important role as a parent of many citrus fruits, such as lemons, oranges and grapefruits. Among the five pummelo accessions, the genetic similarity ranged from a 0.87 to 0.97. This in line with other published reports (Corazza-Nunes et al., 2002; Uzun et al., 2010).

In conclusion, our study reveals the antibacterial activity of natural lemon and lime juices against human pathogenic bacteria, nevertheless further studies are needed to identify the chemical composition of different bioactive compounds containing juice and declare their relation to their antibacterial properties. Our study also, indicated the presence of high genetic diversity among different *Citrus* species and groups currently cultivated in Egypt, however a high level of genetic similarity was detected within each citrus group.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors acknowledge FAO for providing funds for the molecular characterization research

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Supplementary Tables

Table S1. Number of accessions per citrus group, total number of amplicons, monomorphic amplicons and polymorphic amplicons as revealed by 13 ISSR primers.

Citrus group	Number of accessions	Total amplicons	Monomorphic amplicons	Polymorphic amplicons
Sweet orange	20	142	63	79
Mandarin	6	164	69	95
Lemon and lime	10	167	48	119
Sour orange	3	114	93	21
Grapefruit	4	126	88	38
Pummelo	5	139	72	67

Table S2. ISSR positive markers that differentiated *Citrus* species.

Primer	*Citrus* species					
	C. sinensis	***Citrus spp.***	***C. limetta***	***C. jambhiri***	***C. aurantifolia***	***C. aurantium***
	ISSR fragment size (bp)					
H_{12}	408, 500, 764, 1305	-	-	-	-	1233
H_{13}	159, 209	-	-	1024, 1302	-	479, 648, 869, 1734
H_{14}	301, 648	-	-	-	-	836
H_{15}	308, 470, 787	477	-	-	-	798
H_{16}	315	-	-	-	-	-
H_{21}	161	-	-	665	1128	416, 443, 537, 754, 912, 1395, 1714, 2115, 2823, 3470
H_{29}	-	-	-	-	1684	646
P_2	1457	1016	536	-	-	661, 812, 1485
P_3	-	-	-	-	-	368, 439, 599, 893
P_4	265, 1248	-	-	-	312, 368	239, 415, 498, 674, 806
P_{11}	198, 292, 913, 1086	798	-	-	-	435, 1174
P_{16}	308, 470, 787	-	-	-	-	798
Total	23	3	1	3	4	33