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CHEMICAL, BIOLOGICAL AND MOLECULAR STUDIES ON DIFFERENT *CITRUS* SPECIES WASTES

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Abstract

Waste products have a special place in the world of pharmaceuticals these days. So, this study investigated PCR based DNA fingerprinting in a set of five species of Citrus wastes in addition to some biological activities. ISSR markers were presented in dendrogram to address the relationships between *Citrus* species. The volatile oils extracted, were analyzed by GC/MS to identify their constituents which reveal similar constituents with two compounds not common. The oils when examined for its antimicrobial activity against different pathogenic bacteria and fungi indicated that, grapefruit peel oil showed maximum activity followed by lemon leaves oil. Also, it was found that the lemon peel oil induced an cytotoxic activity against two carcenoma cell lines, liver and breast cancer. Citrus wastes could be considered as one of the important sources for drug discovery.

***Keywords*:** *Citrus* species, Plant wastes, ISSR, DNA fingerprinting, Antimicrobial activity, Cytotoxic activity.

Introduction

Agriculture crops (fruits) produce large amount of wastes or by-products every year. These wastes included tons of pruning materials and wastes of juice production of different nutritional industrial companies.

Large amount of waste products is generated after the processing of fruits, such as citrus, bananas, mangoes which contain valuable compounds. The global production of different types of citrus fruits in the year 2016-2017 for orange, tangerine, mandarin, grapefruit and lemons are 50.186, 28.5, 934, and 7209 million metrics tons, respectively (USDA, 2017). Many reports are published related to the recovery of fruit waste as a natural value added compounds such as fiber, bioactive compounds like flavonoids (Casquete *et al.*, 2014; Mossa *et al.*, 2015), additive and colorants (Sharma *et al.*, 2017).

In Egypt, the total cultivated area of citrus trees is 541723 Fadden (4200 m^2) with fruit production of about 4.10 million tons. The waste represents about 2.5% of the total fruit production, which means 1.025 million metric tons of wastes from citrus fruits (Anonymous, 2015).

The common uses of these wastes are as animal feed or compost used to enhance soil fertility instead of chemical fertilization. Their wastes contain the pruned leaves and stems removed after pruning process of fruit trees and different fruit peel after extraction of juice.

Based on the published work performed and from the realm of genus Citrus revealed different phytochemical components e.g. limonoid, triterpenes, limonenea hydrocarbon and Citrus volatile oils. Rajaswari (2015) concluded that *C. sinesis* plant acts as potential source of the antimicrobial agent in his experiment evaluating volatile oils of these species. Azar *et al.* (2011) analyzed the volatile oil obtained from *C. sinensis* var. *valencia*. They recorded the main constituents as limonene 61.34, myrecene 17.55, sabinene 6.50 and α-pinene 6.65%.

In Greece, Kanaze *et al.* (2009) studied the flavonoid content of different methanolic extracts of navel orange peel (*Citrus sinensis*) and assessed its antioxidant activity *in vitro*. The isolated flavonoids were chemically determined by their retention times and spectral data, then verified by detailed LC-DAD-MS analysis. The main isolated flavonoid from this species was polymethoxylated flavonoid. The quantitative HPLC analysis confirmed that hesperidin is the major flavonoid glycoside found in orange peel, and its higher percentage permits the commercial production use of orange peel as a source of hesperidin production.

The aqueous extract of *Citrus limonum* pulp contained carbohydrates, alkaloids, tannins, fixed oils, proteins, cardiac glycosides, sterols, phenols and flavonoids (Mathew *et al.*, 2012).

When Citrus juices were considered, Oikeh *et al.* (2016) reported that juices of *Citrus tangarins, Citrus paradise*, *Citrus limon* and *Citrus aurientifolia* contained: alkaloids, flavonoids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars in all juices concentrates. This investigation suggests that these juices may have beneficial antimicrobial roles that can be exploited in controlling unwanted microbial growth. Ngele *et al.* (2014) from Nigeria reveal the same findings.

Limonoid a triterpenoid group was reported by Senevirathne *et al.* (2009), and Miyake *et al.* (1997) to occur in large amount in Citrus juice and tissues as water-soluble glycoside and found in seeds as water insoluble aglycones. These limonoids are responsible for a wide variety of therapeutic properties (Ma *et al.*, 2008). The potential of

Citrus Limonin and nomilin topical application showed 60% reduction in tumer borden, however nomilin is less effective. Limonin as glycoside or aglycone when administered *in-vitro* to estrogen dependent or independent human, breast cancer cell line proved equal activity like the standard drug tamoxifen. These limonoid, is more potent than tamoxifen for its activity against estrogen undependant cancer cells (Jacob *et al.*, 2000).

Therefore, the present study was conducted to investigate the anticancer and antimicrobial activity of volatile oils of five species of Citrus wastes as Lemon, Orange, Valencia, Navel, mandarin, and Grapefruit. GC/MS analysis and molecular studies by using PCR based DNA fingerprinting in a set of using ISSRs were used.

Material and Methods

Plant Materials

Citrus waste as pruning materials were collected from a private farm on February 2017. The pruning wastes were collected from *Citrus sinensis* trees (Navel), *Citrus sinensis Valencia. Citrus lemon*, *Citrus grandise* (grapefruit) and *Citrus delisiosa* (mandarin). Fruits of each Citrus species were collected from March to April. The peels were removed, weighed as fresh and air dried. The pruning wastes composed of leaves and small stems were freshly weighed then oven dried at 45°C and again weighed. The fresh pruning wastes were water distilled using Clevenger apparatus to determine volatile oils percentage in each species. Fresh peel of each Citrus fruits was also hydrodistilled to estimate volatile oil percentage. In the same time large amount of wastes were hydrodistilled to collect higher amount of volatile oils for studying the differed biological activities (Diab *et al.*, 2018).

Cytotoxic effect on human cell lines

The cytotoxic activity of essential oils collected from studied citrus species were examined against two cell lines; HepG2 (liver cancer) and MCF7 (breast cancer) cell lines. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983) the experiment was done according to standard protocol and condition (El-Menshawi *et al.*, 2010). A positive control which composed of 100µg/ml was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions (Thabrew *et al.*, 1997; El-Menshawi *et al.*, 2010). DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%.

A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The percentage of change in viability was calculated according to the following formula:

(Reading of extract/ Reading of negative control) -1 × 100

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

Antimicrobial activity

Ten different plant oils for leaves and peel from the five Citrus species were used to check their activity as antimicrobial.

(i) Microbial strains and media

Four bacterial strains and one fungal strain of significant importance were used to test the antibacterial properties of the plant oils. Two of bacterial strains were Gram positive (*Staphylococcus aureus* NRRL B-313 and *Bacillus cereus* NRC) the others were Gram negative (*E. coli* NRC B-3703 and *Pseudomonas aeruginosa* NRC B-32) while the fungal strain was *Aspergillus niger* NRRL 599. Nutrient agar media was used in this study for bacterial strains growth and malt extract-agar (MEA) according to Dutton and Penn (1989)**.** The typical formula (g/L) was: malt extracts 30.0; peptone 5.0; agar 15.0 was used for fungal strain growth. The liquid medium was sterilized by autoclaving at 121°C for 20 min and then used for subculture then solid media was used for agar-well diffusion assay.

(ii) Well diffusion assay

The well diffusion method was used to evaluate the antimicrobial activity of different species essential oils. Ten mL of an agar medium (nutrient and malt) was poured into sterile Petri dishes followed with 15 mL of a seeded medium previously inoculated with the bacterial suspension to attain a 10^5 CFU/mL of medium. These microorganisms were cultured and incubated at 37°C for 24 h. The inoculums' suspension was spread uniformly over the agar plates using a spreader, for a uniform distribution of bacteria. Subsequently using a sterile borer, a well of 6 mm diameter was made in the inoculated media then 50 µL of essential oil each was added. The plates were kept in the fridge at 4°C for 2 h to permit the essential oil diffusion. They were incubated at 35°C for 24 h. The presence of inhibition zones was measured and considered an indication of antibacterial activity **(**Dutton and Penn, 1989**).**

(iii) Determination of Minimum Inhibitory Concentrations (MIC)

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after twenty-four hours of incubation on the agar plates. The most effective seven essential oils which exhibited a strong antibacterial activity were manipulated to determine their MIC using the well diffusion method. Different concentrations of the effective essential oils (5-50 µL) were prepared separately, sterilized through Millipore filter and their requisite amount was loaded into well (6 mm in diameter). Agar was poured into the sterile Petri dishes seeded with the microbial suspensions of the pathogenic strains. The plates were kept in the fridge at 4°C for 2 h, and were then incubated at 35°C for 24-48 h.

Molecular marker investigation

Molecular Analysis of the five Citrus species included in the present investigation was studied.

(i) DNA Isolation

Leaves of the five commercially important Citrus species were collected and soaked in liquid nitrogen for DNA extraction. The DNA was extracted by Cetyl Trimethyl-Ammonium Bromide (CTAB) method (Murray and Thompson, 1980).

(ii) Polymerase chain reaction (PCR) procedure

ISSR analysis was performed as described by Adawy *et al.* (2004) and Hussein *et al.* (2006). Six ISSR primers were

selected from different nucleotide sequences (Table 1). Gels were photographed and scanned with Bio-Rad video densitometer model 620, at a wavelength of 577 nm.

Data analysis

The bands detected by photo Capt MW program and calculation were achieved using Dice similarity coefficients (Dice, 2004). The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among species as revealed by dendrograms were done using SPSS program version 16.

Table 1 : List of the primer names and their nucleotide sequences used in the study for ISSR procedure.

No	Name	Sequence
1	14A	5 CTC TCT CTC TCT CTC TTG 3`
2	44B	5` CTC TCT CTC TCT CTC TGC 3`
3	98A	5´ GAG AGA GAG AGA CC 3`
4	99A	5´ CGC TAC CTC TC 3`
5	HB- 12	5´ CAC CACCAC GC 3`
6	HB-15	5´ GTG GTGGTG GC 3`

Results and Discussion

Volatile oils of *Citrus* spp.

Citrus wastes emerged from pruning process were subjected to hydrodistillation using Clevenger apparatus for 3 h after boiling. The oil obtained was measured as mL and the percentage was calculated as recorded in following Table (2). The results revealed that the five Citrus wastes varied in their volatile oil percentage. In pruning wastes, the higher oil percentages were obtained from *Citrus lemon* pruned material (0.56) then from mandarin pruning waste (0.53). On the other hand, the lowest percentage was obtained from grape fruit pruning wastes (0.07).while, peel of the fruits (fresh) showed also variable oil percentage, the highest oil percentage was obtained from grape fruit peel (0.66), followed by the percentage of volatile oil obtained from orange peel (Navel) fruit (0.451), however *Citrus lemon* peel produced the lowest percentage.

Table 2 : Oil percentage of different Citrus wastes.

Citrus species	Oil % in fruit peel	Oil % in leaves
Citrus sinensis peel (Navel)	0.45	0.23
*Citrus sinensis (*valentia)	0.41	0.37
Citrus grape fruit	0.66	0.07
Citrus lemon	0.051	0.56
Citrus Mandarin	0.42	0.53

The volatile oil constituents analyzed by GC/MS were recorded in Table (3). Results revealed that, Navel orange leaves pruning waste's volatile oil contain sabinene, D-limonene and 3-Carene as major compounds in the following percentages 23.56, 10.43 and 13.59, respectively. Peel oil of Navel orange fruit contains 6 compounds only with D-limonene amounted 90.20%, β-myrecene 4.3% and valencene 1.31% which occurred in peel only. Lemon oil was represented by two samples, leaves and peel. Leaves oil contains sabinene 29.5, carene, 7.18, limonene 7.86 and ocemine 8.27, however the peel's oil resolutes to D-limonene in a percentage of 45.67, δ-terpinene 13.51, α-pinene 11.11 and β-bisabolene 3.53; which is a sesquiterpene.

The leaves and peel fruit oils of the third Citrus species is grape fruit. Grapefruit leaves oil contain sabinene, D-limonene, β-ocimene, Terpen 4-01 and Trans caryophyllene in the ratioes of 20.25, 4.12, 10.23, 6.08 and 4.93 respectively.

Grapefruit peel volatile oil composed of D-limonene with percentage of 71.96, β-myrecene 2.59, and octanal amounted to 3.06, which is completely different from leave's oil.

Leaves of Valentia orange oil did not differ greatly from Navel leaves oil in its constituents. Sabinene, carene, β-ocimene, citral, its esters, and elemene were the higher constituents found, resembles nearly the same as Navel orange but with variable percentage.

Valentia peel oil contains nearly the same constituents like leaves, but with higher limonene and neryl acetate. Appreciable amount of sequiterpenes were also found in peel oil.

Volatile oil of mandarin leaves contains dimethyl anthranilate of 64.78% a compound found only in this Citrus species beside the other constituents commonly found in the other species. Interstingly mandarins peel oil show another picture which looks like the other Citrus volatile oil. Peel oil decomposed of limonene in a proportion of 65.50% and δ-terpinene of 18.94%, and no sesquiterpenes.

These finding go parallel with those stated by Viuda-Martos *et al.* (2009) to some extent and are parallel also with the finding obtained by Russell and Perez-Cacho (2007).

In conclusion to this part all the volatile oils, extracted from different Citrus parts of the different Citrus species have nearly the same constituents, with variable concentrations. The esters found in the analyzed oils varied with species. However, there is a compound or more that are characteristic to species. These specific compounds may be an important signal or marker for that oil and may be used as a tool in chemotaxonomic investigation.

The five analyzed Citrus oils; limonene was a common factor and the most abundant component. However, the great variability may be due to several factors, among them the particular variety studied, the geographical location, season, plant part and environmental factors.

Table 3 : The constituents of the essential oil of *Citrus sp*.

Rt	Constituent	Concentration (%) Navel		Concentration (%) lemon		Concentration (%) grapefruits		Concentration (%) Mandarin		Concentration (%) Valentia Orange	
		Leaves	Peel	Leaves	Peel	Leaves	Peel	Leaves	Peel	Leaves	Peel
5.74	2-hexane E	0	0	0	0	0.49	0	0	0	0	0
7.94	Bicyclors	1.35	0	0.1	1.36	0	0	0	0.96	0.88	0
8.14	α-pinene	4.1	0	3.52	0	0.48	0	0	0	0	0
9.49	Sabinene	28.4	0	29.5	2.68	20.25	0.85	0	2.08	22.33	0.34
9.67	β-pinene	0	1.65	0	0	1.66	0	2.95	0	0	0
9.7	2-β-pinene	1.98	0	1.9	2.2	1.48	2.34	11.11	2.54	1.46	3.21
10.16	β-myrecene	4.32	4.3	3.96	0	2.31	2.59	1.64	2.3	3.49	0.41
10.56	α-phyllandrene	0.92		0.81	0	1.51	0	0.74	0	0.96	
10.61	Octanal	0	0	0	0	0	3.06	0	0	0	0
10.85	3-carene	7.33	0.91	7.18	0	0	0	0	0	9.5	0
11.0	α-terpinene	2.4	0	1.61	0	0	0	13.51	0	1.42	0
11.33	O-cymene	0.38		0.35	4.79	0.35		0.62	0	0.54	2.72
11.43	D-limonene	7.81	90.02	7.86	6.06	4.12	71.96	45.67	65.5	0.46	19.35
12.19	β-ocimene	8.31	0	8.37	0	10.23	0	0	0	9.99	0
12.52	ϑ-Terpinolene	2.85	0	2.48	0	0.94	0	1.39	0.94	2.69	0.31
12.54	ϑ-terpinene	3.52	1.81	2.64	16.67	3.04	0	0	18.94	2.13	9.2
12.8	Trans-sabinene hydrate	0.64	0	0.62	0	0.52	0	0	0	0.55	0
13.51	Cyclohexane, 3-methyl, 6-1 methydiene	0.35	0	0.32	0	0	0	0	0	0.39	0
13.58	α-Terpinoline	0	0	0	0.75	0	0.87	0	1.55	0	0.87
14.03	Linalool	1.32	0	1.38	0	4.94	2.45	1.36	0	7.95	2.45
14.23	Cyclohexane, 1-methyl, 1,3 4trimethyl 5	0	0	0	0	0.36	0.31	0	0	0	0.31
14.6	Linalool oxide	0	0	0	0	0.22	0	0	0	0	0
14.7	Cyclohexane 1-01-1-methyl	0.4	0	1.19	0	0.38	0	0	0	0.25	0
15.9	Citronellal	1.18	0	4.45	0	2.07	0	0	0	4.12	0
16.7	Terpene 4-01	6.13	0	1.39	0	6.08	0	1.64	0	4.04	4.59
17.23	α-terpineol	0.42	0	0.37	0	0.34	1.6	3.26	0	0.42	6.21
18.58	Citronellol	1.48	0	0.56	0	0.27	0.52	0	0	1.36	0.52
18.70	Nerol	0	0	0	0	0	0	1.11	0	0	2.91
19.02	Z-citral	0.49	0	0.61	0	0.6	0.74	1.17	0	2.03	9.9
19.60	Geraniol	0.63	0	0.65	0	0	0	1.17	0	0.48	2.94
20.05	E-citral	0.56	0	0.37	0	0.75	0.9	1.9	0	2.38	12.34
21.83	Trans geranic acid methyl ester	0	0	0	0	0	0	0	0	0.28	0
22.81	Citronellyl acetate	0.39	0	3.71	0	0.24	0	0	0	1.07	0
23.17	Neryl acetate	0	0	0	0	0	0	1.24	0	0.63	4.79
23.5	α- Copaene	0	0	0	0	0	0.9	0	0	0	0
24.05	β-elemene	3.2	0	5.18	0	0.44	0	0	0	3.35	0.22
24.56	Dimethyl anthranilate	0	0	0	64.78	0	0	0	0	0	0
24.90	Trans caryophyllene	1.6	0	0.72	0.72	4.93	0.9	1.53	0	1.17	1.21
25.31	Benzene 1, methyl 3, (1-methyl etheyl)	0	0	0	0	0	0	0	2.37	0	0
25.4	Cis alpha bergametene	0	0	0	0	0	0	2.23	0	0	0
25.42	Trans m-bergamotine	1.97	0	0	0	0	0	0	0	0	0
25.9	Humulene	0.61	0	1.4	0	1.69	0	0	0	0.62	0
26.07	Cis-beta Farnesene	1.16	0	0	0	2.12	0	0	0	0.83	0
26.45	Methyl anthranilate	0	0	1.98	0	0	0	0	0	0	0
26.81	Germacrene D	0	0	0	0	0	0.9	0	0	0	0
27.18	Valencene	0.45	1.31	0.37	0	1.16	0	0	0	6.94	1.31
27.27	15, 2E, 6E, 10R	0	0	0	0	1.51	0	0	0	0	0
27.66	beta bisabolene	0	0	0	0	0	0	3.53	0	0	4.95
28.09	Delta cadiene	0	0	0	0	0	0.9	0	0	0	0
29.10	1,5, cyclodecadiene	0	0	0	0	0	0	0	0	0	0.45
29.3	CH-Inlol-z-one- (2-6..)	1.57	0	0	0	1.32	0	0	0	0	0
29.7	Spathulenol	0	0	0	0	0.37	0	0	0	0	0
29.89	Caryophyllene oxide	0	0	0	0	0.57	0	0	0	0	0
32.67	Ledol	0	0	0	0	0	0	0	0	0	0.81
32.71	Seline -6-en-4-alpha-01	0	0	0	0	0.52		0	0	0	0
33.41	Isolongifoline	0	0	0	0	0	0	0	0	0	1.02
34.20	α-bisabolol	0	0	0	0	0	0	0	0	0	1.11
34.90	2,6,11, dodecatrienal 2,6 dimethyl, 10 methylene	0	0	0	0	5.29	0	0	0	0	0
34.91	2,6, 11-dodecatrienal	2.5	0	2.62	0	0	0	0	0	1.27	0
37.4	2,6,9,11 dodecatrienal	0.89	0	0.98	0	0	0	0	0	0.69	0
39.33	Nootktone	0	0	0	0	0	0.9	0	0	0	0.34
39.94	Hexadecanal	0	0	0	0	0	0	0	0	0	0.27
44.76	n-hexadecanoic acid	0	0	0	0	0	0	0	0	0	0.46
48.73	Phytol	0.33	0	0	0	6.91	0	0	0	0.26	0

Cytotoxic effect on human cell lines

The literature revealed that plants are containing many secondry metabolites which possess several biological activities (Hassan *et al.*, 2016; Hassan *et al.*, 2015; Hassan *et al.*, 2008)

different biological activities for Citrus wastes studied. Volatile oils found in the wastes with appreciable amounts, make it important to investigate its biological activities as cytotoxic and antimicrobial.

Jacob *et al.* (2000) studied the antitumor activity of limonen and nomilin, they reported 60% reduction in tumer borden, however nomilin is less effective.

Also, the limonoids triterpenes were reported by Senevirathine *et al.* (2009) to occur in large amount in Citrus juice and tissue as water soluble glycoside and found in seeds as insoluble aglycone. These limonoids are responsible for wide range of therapeutic activity. The limonoids proved to have equal activity like the standard drug tamoxifen a standard anti-cancer drug (breast cancer).

Based on the GC/MS analyses all Citrus volatile oils contain limonene, ɤ-terpinene, α-pinene, and β-myrcene which reported by Odeh *et al.* (2012) to have cytotoxic effects. So, these oils were investigated for its cytotoxic activity against two cell lines; liver HepG2 human hepato cellular carcinoma and MCF7 human Caucasian breast adino carcinoma cell line. The results obtained from the cytotoxic studies reveal that the IC_{50} for the volatile oil obtained from leaves of grapefruit is 51.2 ug/ml and 65.5 ug/ml which induced 50% inhabitation of breast cancer cell line and $HepG_2$ cell line, respectively. These results indicate a week activity against the two cancer cell lines used. Lemon peel oil produced a week activity on the two cell lines mentioned before. The IC_{50} of thisoil is 71.8 ug/ml and 81.5 ug/ml that induced 50% inhibition on the two studied cell lines, respectively. The other tested volatile oils did not show any activity against the two cell lines investigated and this did not coincide with that proved by Jacob *et al.* (2000).

Antimicrobial investigation

Plant essential oils and extracts have been used for many thousands of years (Jones, 1996), in food preservation, pharmaceuticals, alternative medicine and natural therapies (Reynolds, 1996; Lis-Balchin and Deans, 1997). Essential oils are potential sources of novel antimicrobial compounds (Mitscher *et al.*, 1987) especially against bacterial pathogens. In vitro studies in this work showed that the essential oils inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of many essential oils has been previously reviewed and classified as strong, medium or weak (Zaika, 1988).

The antimicrobial activity of the ten selected essential oils against five microorganisms is summarized in Table (4). The results revealed that the selected essential oils showed antimicrobial activity with varying magnitudes. The zone of inhibition above 10 mm in diameter was taken as positive result. Generally, most of the tested organisms were sensitive to many of the essential oils. Out of ten essential oils tested, seven showed antimicrobial activity. *C. sinensis* (Navel orange leaves), *C. limon* (Lemon leaves), *C. sinensis* (Valentia orange leaves), *C. paradise* (Grape fruit peel), *C. sinensis* (Valentia orange peel) and *C. reticulate* (Mediterranean Mandarins peel) oil showed maximum activity against all the microorganisms tested.

Minimum inhibitory concentration (MIC) for the selected seven oils ranged from 5 to 50 µL (Table 5). This study revealed that *C. limon* (Lemon leaves) oil showed maximum activity with MIC values ranging from 20 to 40 µL followed by *C. paradise* (Grapefruit peel) oil with MIC values ranging from 5 to 10 µL against all the tested strains whereas the remaining oils showed moderate MIC values.

Table 4 : Inhibition zone of different plant oils against some pathogenic bacteria and fungi by well diffusion method (Size of well by mm).

Samples		Gram –ve		Gram +ve		Fungi
		P. aeruginosa	***E. coli***	***B. ceruse***	***S. aureus***	***A. niger***
C. sinensis (Navel orange)	leaves	21.5	00	28	13.5	12
	peel	15	00	12.5	10	00
C. limon (Lemon)	leaves	49	00	00	32	49
	peel	00	00	11.5	12.5	00
C. sinensis (Valentia orange)	leaves	9.5	00	17.5	13	00
	peel	17	12	10	19	00
C. paradise (Grape fruit)	leaves	00	00	00	00	00
	peel	24.5	00	17	24.5	11
C. reticulate (Mediterranean Mandarins)	leaves	00	00	14.5	10	14.5
	peel	14.5	10	10	16.5	00

Industrial citrus essences from sweet orange, red orangeade, bitter orange, red orange, sicily orange and sweet lime had shown inhibitory activity against *Saccharomyces cerevisiae* (Belletti *et al.*, 2004), while essential oil from *Citrus sinensis* (L.) inhibited growth of *A. niger*. In addition, the antibacterial activity of essential oils from lemon was reported against various Gram-positive and Gram-negative bacteria (Baratta *et al.*, 1998). Generally, the antimicrobial activity of essential oils is not due to a single mechanism, but to several sites of action at the cellular level. First mechanism is the generation of irreversible bacterial membrane damage resulting in cytoplasmic losses, leakage of ions, loss of energy substrate (glucose, ATP) causing bacterial lysis and death. Another possible mode of action is the inhibition of amylase and protease production, therefore stop the toxin

production, electron flow and cell content coagulation (Burt, 2004; Di Pasqua *et al.*, 2007).

Molecular investigation

Citrus is one of the most important and widely grown fruits crops (Ahmed *et al.*, 2017). *Citrus* L. genus includes orange varieties, lemons, tangerine, mandarins, grapefruits and others [37]. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. An ideal marker should be polymorphic, independent, and reliable, providing sufficient resolution relatively easily, quickly and with fairly low costs (Mabberley, 2008). Development and utilization of marker to detect differences in the DNA of individual plants has many applications in crop improvement in fruits. These differences are known as molecular markers because they are often associated with specific genes and act as "signposts" to those genes (Gosal *et al.*, 2010). In addition, markers and comparative mapping of various species have been very helpful in enhancing our understanding of genome structure and function. Molecular markers have provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters.

Table 5: MIC (µL) of plant oils.

Samples		***St. aureus***	***P. aeruginosa***	***A. niger***
C. sinensis (navel orange)	leaves	<20	<20	50
C. limon (Lemon)		<5	<5	10
C. sinensis (Valentia orange)		30	40	--
C. reticulate (Mediterranean Mandarin)		50	--	50
C. paradise (Grape fruit)	Peel	<20	<20	40
C. sinensis (Valentia orange)		30	40	--
C. reticulate (Mediterranean Mandarin)		30	40	--

Table 6 : DNA amplified bands and polymorphism% generated with the 5 Citrus cultivars using 6 ISSR primers.

Primer code	**Monomorphic bands**	**Unique bands**	**Polymorphic bands**	**Total bands**	**Polymorphism %**
14A	2	4	-	6	66.6
44 B	2	-	1	3	33.3
98 A	2		2	4	50
99 A	2	-	1	3	33.3
HB 12	2	-	4	6	66.6
HB 15	3	1	4	8	62.5
Total	13	5	12	30	56.6

A maximum of 30 DNA bands was scored in the ISSR profiles generated by the 6 ISSR primers. The size of the amplified bands ranged from about 260-1460 bp. These bands were identified as 12 polymorphic bands, 13 monomorphic ones and five unique bands (Table 6 and Figure 1).

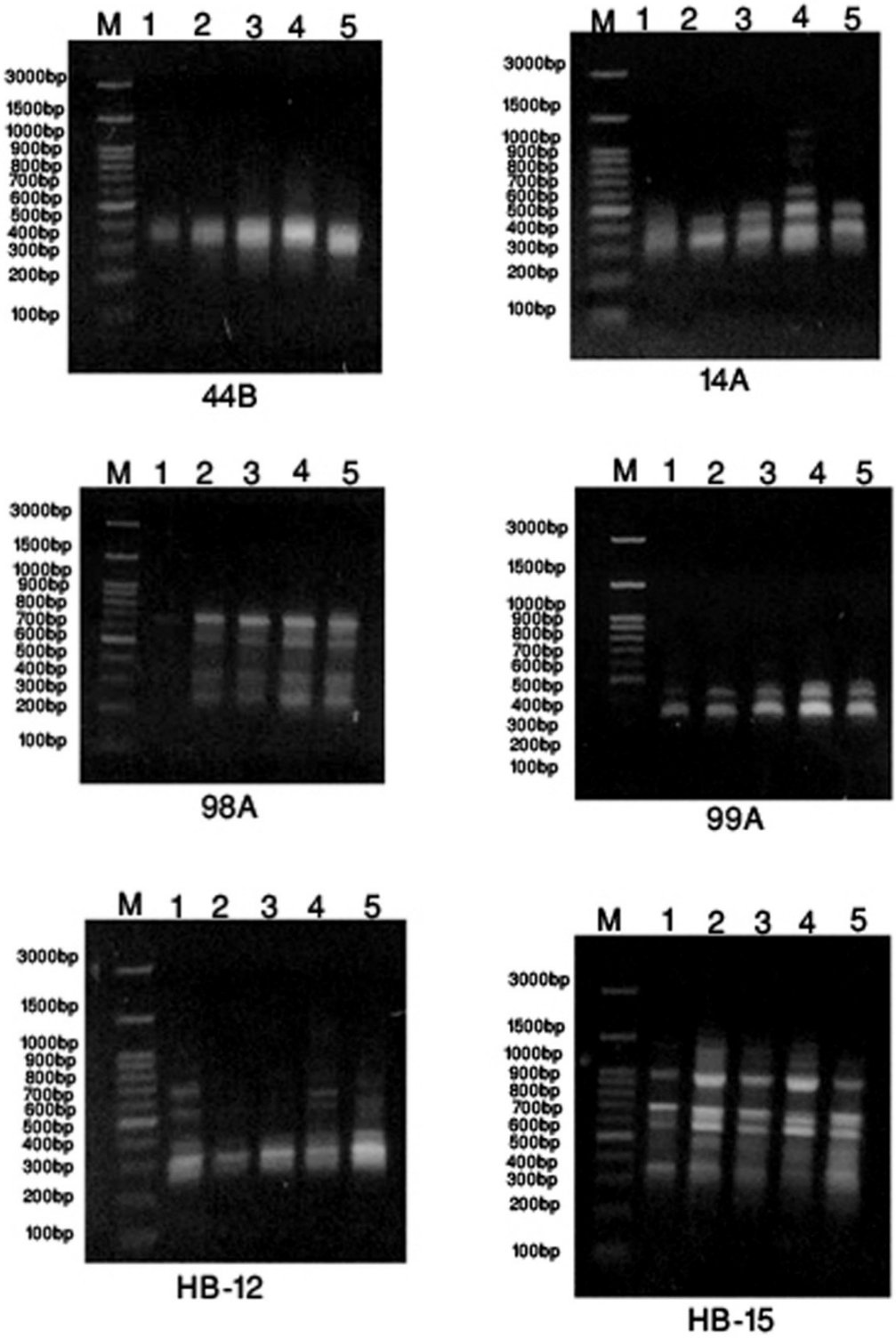
The primer 14 A (Table 6) was generated 4 unique polymorphic fragments and 2 monomorphic fragments. The most pronounced are the 4 unique polymorphic fragments identified at the apparent molecular size of 1230, 1000, 830 and 600 bp were exhibited in mandarin. The 2 monomorphic fragments were scored with all species at molecular size 430 and 360 bp.

Primer 98 A generated 4 bands ranged from 260-700 bp, 2 of them were monomorphic at apparent molecular size 340 and 700 bp as shown Figure (1). The bands with molecular size 260 and 560 bp, were polymorphic bands and founds in all species except in lemon species.

The results of primer 99 A were generated 2 monomorphic bands which were recognized at molecular size 340 and 460 bp and one polymorphic band was appeared at molecular size 500 bp was scored with Valencia, mandarin and grapefruit (Figure 1).

Primer 44 B exhibited 3 bands; 2 monomorphic bands were appeared at molecular size 340 and 400 bp, and one polymorphic band was scored at molecular size 300 bp.

The results of primer HB 12 detected 2 monomorphic bands were recognized at molecular size 260 and 300 bp and 4 were polymorphic. The fragments with molecular sizes 400 and 560 bp were observed with lemon and mandarin. The band exhibited at molecular size 600 bp was scored with mandarin and grapefruit, while the band detected at molecular size 680 bp was appeared with lemon, mandarin and grapefruit (Figure 1).



ISSR Citrus

Fig. 1 : ISSR Profile of 5 *Citrus* species generated (number 1 to 5) by 6 primers, M=DNA Marker

1. *Citrus lemon*.
2. *Citrus sinensis* (Naval orange).
3. *Citrus sinensis* (Valencia)
4. *Citrus delesiousa* (mandarin).
5. *Citrus grandise* (grape fruit).

The results of ISSR analysis were pooled together to generate the dendrogram (Figure 2). The five *Citrus* species were divided into two main groups. The first include mandarin only but the second was divided into two main sub groups. The first involved lemon only but the second includes Navel orange, Valentia orange and mandarin. The second sub group divided into two main classes. The first includes Navel and Valentia orange, the second class involved grapefruit only.

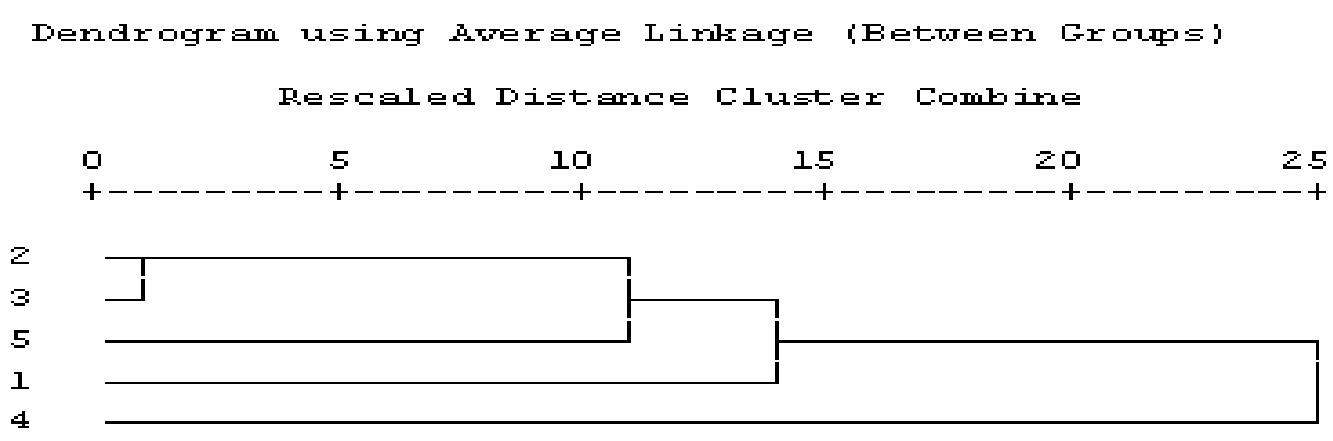


Fig. 2 : Dendrogram illustrating genetic distance between 5 citrus species based on ISSR data.

Similar results were obtained by many authors; Fang *et al.* (1998) study phylogenetic relationships among 46 *Citrus* L. accessions representing 35 species by ISSR. ISSR markers have successfully been used in *Citrus* to identify closely related varieties Fang and Roose (1997), to determine genetic diversity, characterization, assess phylogenetic relationships among the *Citrus* and related genera (Fang *et al.*, 1998; Gulsen and Roose, 2001a, 2001b; Shahsavar *et al.*, 2007; Marak and Laskar, 2010) and to fingerprint and group trifoliate accessions (Fang and Roose, 1997). De Pasquale *et al.* (2006) characterized 5 sour orange clones using ISSR markers by using 11 primers and reported clearly distinct patterns among the clones. The high grade of polymorphism was showed from AACNR 32 clone. It fits very well with the particular morpho-physiologic character shown by this plant and confirms its supposed natural hybrids. Uzun *et al.* (2010) distinguished 29 grapefruit (*Citrus paradisi* Macf.), 5 pummelo (*Citrus maxima* (Burm.) Merr.) and 1 *Citrus* hassaku Hort. ex Tanaka accessions by using ISSR markers.

Conclusion

The results obtained recorded the following conclusions: the cytotoxic activity investigation will be performed on the extracts obtained because a weak activity was obtained with the volatile oil. Maximum antimicrobial activities against some pathogenic bacteria and fungi were obtained by seven volatile oils from the ten oils tested and this will be further studied to attribute the effects obtained to the chemical compounds found in the oils. ISSR markers is an important tool which successfully identify the closely relationship between *Citrus* species and determine genetic diversity.

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Conflict of interest

There no Conflict of interest

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