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Correlation Between Genetic Variability, Chemical Composition and Antimicrobial Activity of Essential Oils Isolated from Avocado Cultivars Grown in Egypt

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Abstract

Persea americana Mill. (Lauraceae) has been successfully propagated in Egypt for the commercial production of avocado fruits. Four cultivars (one pure Mexican and three Mexican-Guatemalan hybrids) were investigated for the chemical composition and antimicrobial activity of their leaves' essential oils. The hydro-distilled oils from Bacon, Duke, Ettinger and Pinkerton cultivars were analyzed using Gas Chromatography/ Mass spectrometry (GC/MS) and Gas Chromatography/ Flame Ionization Detector (GC/FID). Total of 20 compounds were identified with estragole, methyl eugenol and α-pinene as the major constituents. Chemometric analysis performed on the GC/FID data indicated close similarity between the essential oils of Bacon and Ettinger cultivars, while that of the Duke cultivar was the most distant. This classification was further supported by comparative DNA fingerprinting using Random Amplified Polymorphic DNA (RAPD) technique. Essential oils of all cultivars had no antifungal activity against *Candida albicans* or *Aspergillus niger* but showed good antimicrobial activities against *Streptococcus mutans* and *Salmonella typhimurium* with IC_{50} values between 0.4 and 20 µL/mL

Keywords: Avocado; Essential oil; GC/MS; Streptococcus mutans; DNA Fingerprinting, Estragole

1. Introduction

Avocado trees (*Persea americana* Mill.) have originated from Mexico around 500 B.C. but are now cultivated worldwide for commercial production of avocado fruits. All avocado cultivars are derived from three horticultural races: Mexican, Guatemalan, and West Indian [1]. Some cultivars have been successfully cultivated in the Middle East region including Egypt. Avocado is widely used as a healthy diet due to its high content of vitamins, antioxidants and unsaturated fatty acids [2, 3]. Many secondary metabolites such as phytosterols and carotenoids were also identified in different organs of avocado tree including leaves, seeds, fruit pulp and peel [4].

Avocado leaves have a characteristic odor implying the presence of essential oil, however the yield of the essential oils and hence the intensity of leaf aroma may vary greatly according to the cultivar [5, 6]. Constituents previously identified in the essential oils of avocado leaves include estragole, *α*- and *β*-caryophyllene, *α*- and *β*-pinene, germacrene, methyl eugenol and anethole [5, 6]. The chemical composition of the leaf essential oil was found to vary widely among different cultivars and geographical areas [6, 7].

Some of the constituents identified in the avocado leaves' essential oils were found to exert antibacterial and/or antifungal activity including estragole [8], methyl eugenol, *α*-pinene and *β*-pinene [9]. In addition, estragole was known to act as anxiolytic, anti-inflammatory and smooth muscle relaxant [10, 11].

Reports on the essential oils' composition of avocado trees growing in Egypt are scarce and no comparative study concerning the different cultivars has been traced. Thus, three Mexican-Guatemalan hybrids (Bacon, Ettinger and Pinkerton) and a pure Mexican cultivar, Duke, introduced in Egypt were selected for this study. Chemical composition and antimicrobial activity of the essential oils from these cultivars were

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investigated. These results were further analyzed in correlation to a comparative DNA genotyping of the four cultivars that was performed using Random Amplified Polymorphic DNA technique (RAPD).

2. Experimental

2.1 Plant material:

Leaves of *Persea americana* Mill. Cultivars, Bacon, Duke, Ettinger and Pinkerton, were collected from plants grown at the Horticulture Research Institute. Plant specimens were deposited at the Herbarium of the Pharmacognosy department, Cairo University.

2.2 Microorganisms:

Streptococcus mutans (RCMB 010018), *Staphylococcus aureus* (RCMB 010027), *Salmonella typhimurium* (RCMB 010072), *Pseudomonas aeruginosa* (RCMB 010043), and *Escherichia coli* (RCMB 010056) were grown in Mueller Hinton broth. Antifungal activity was tested against two fungi: *Aspergillus niger* (RCMB 62565), and *Candida albicans* (RCMB 05035) which were grown in Sabouraud dextrose broth.

2.3 Preparation of essential oil:

Mature leaves (300 g) were subjected to hydro-distillation for 3 hours using Clevenger-type apparatus. The distilled oils were collected and dried over anhydrous sodium sulfate, filtered and stored at -20°C till time of analysis. The essential oils were obtained as yellowish color liquid lighter than water with anise like odor.

2.4 Gas chromatography/Mass spectrometry (GC/MS):

The analysis by GC/MS was performed using a Shimadzu GC-17A gas chromatograph equipped with a DB5-MS fused silica capillary column (30 m x 0.25 mm; film thickness 0.25 μm) and coupled to GC/MS-QP5050 mass analyzer. Operating conditions were: carrier gas He, flow 0.9 mL/min; oven temperature program: 40-240°C at 3°C/min; sample injection port temperature 240°C; detector temperature 230°C; ionization voltage and ionization current were set according to tuning results; scanning speed 0.5 s.;and split ratio of 1:54. Essential oil components peaks were first deconvoluted using AMDIS software (www.amdis.net).

2.5 Gas chromatography coupled to flame ionization detector (GC/FID):

The oils obtained from the leaves of *Persea americana* cultivars were analyzed by GC/FID using an Agilent 6890 gas chromatograph. The data were obtained on a 5% phenyl- 95% methyl siloxane fused silica capillary column (30 m x 0.32 mm; film thickness 0.25 μm), Agilent 119091J-413. Operating conditions were: carrier gas He, flow 1.0 mL/min; oven temperature program: 70-325°C at rate of 8°C/min (20 min); sample injection port temperature 220°C; detector temperature 250 °C; split ratio 1:75.

2.6 Peak identification:

Compounds were identified by their Kovats indices (KI) relative to n-alkanes (C6-C20) and through matching mass spectra and retention indices with those deposited in the NIST, WILEY library database and reported in the literature. Concentration expressed as percentage was obtained through the integration of the total FID chromatogram, expressed as peak area percentage. Integration of GC/MS data using ion intensity of the base peak was also used to verify quantitative compositional data.

2.7 RAPD analysis:

DNA was extracted from fresh leaves using the Qiagen DNeasy kit (Qiagen Santa Clara, CA). PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR system 9700 (PE Applied Biosystems). Amplification products were resolved by electrophoresis and visually examined and scored for the presence (1) or absence (0) of DNA bands. The similarity matrix was obtained through the cluster analysis of data using Unweighted Pair Group Method using Arithmetic Average (UPGMA).

Ten Primers purchased from Operon Technologies Inc. (Almeda, California, USA) were used for Random Amplified Polymorphic DNA (RAPD) analysis. Primers' sequences are shown in Table 1.

Table 1: Sequence of ten decamers used in RAPD analysis

1.	OPA-06: 5’-GGTCCCTGAC-3’
2.	OPA-10: 5’-GTGATCGCAG-3’
3.	OPA-13: 5’-CAGCACCCAC-3’
4.	OPA-15: 5’-TTCCGAACCC-3’
5.	OPA-16: 5’-AGCCAGCGAA-3’
6.	OPA-17: 5’-GACCGCTTGT-3’
7.	OPB-12: 5’-CCTTGACGCA-3’
8.	OPC-11: 5’-AAAGCTGCGG-3’
9.	OPG-06: 5’-GTGCCTAACC-3’
10.	OPG-08: 5’-TCACGTCCAC-3’

2.8 Antimicrobial assay:

Broth dilution method was used for the determination of minimum inhibitory concentration (MIC) and IC_{50} of the essential oils from each cultivar. Serial dilutions of each oil were prepared and mixed with broth media

in 96-wells microtiter plate to achieve a final concentration range from 0.003 to 4 % v/v. The plates were then inoculated with standardized suspension containing 5×10^5 bacterial count per well. Optical density at 600 nm was determined after specified incubation period by using Jenway 6051 colorimeter (U.K.) and was used as a measure of bacterial growth [12].

3. Results and discussion

3.1 Composition of the essential oil

Essential oils prepared by hydrodistillation from the dried leaves of four avocado cultivars grown in the same latitude were analyzed by both GC/MS and GC/FID. Duke cultivar produced the highest yield of essential oil at 1% v/w, while the Pinkerton cultivar had the lowest yield at 0.25% v/w. Meanwhile, the essential oil yields from Bacon and Ettinger cultivars were approximately similar, about 0.4%. GC/MS and GC/FID analysis allowed the identification of 18, 13, 15, and 15 compounds amounting for 91.22%, 98.98%, 97.16%, and 96.4% of the total oils from Bacon, Duke, Ettinger, and Pinkerton cultivars, respectively (Table 2).

Estragole was the single major constituent (present at 93.5 %) in the essential oil from the pure Mexican cultivar, Duke. Interestingly, the decrease in estragole percentage obviously correlated with that of the Mexican component of hybrid cultivars. In fact, among the analyzed samples, that of Pinkerton cultivar (the predominately Guatemalan hybrid) showed the lowest estragole content (29.5%) and the highest ratios of hydrocarbons represented principally by α-pinene, β-pinene and β-caryophyllene (Table 2). Hierarchical cluster analysis of the results showed the pure Mexican Duke cultivar as the most distant branch separating it from the three hybrid cultivars.

3.2 RAPD analysis

To correlate variation in the chemical composition of essential oils of these cultivars to their genetic make-up, RAPD analysis was performed using 10 decamers [13]. Results from RAPD analysis determined that the highest similarity is estimated at 95 % between Bacon and Ettinger cultivars, Table 3. Meanwhile, Duke cultivar showed the lowest similarity, as seen in table 3, in agreement with the chemical profile of its essential oils (Figure 1).

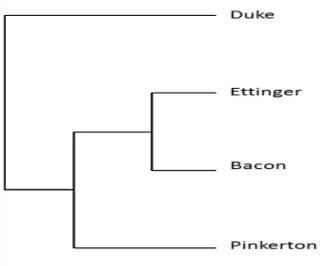

Figure 1: Hierarchal cluster analysis based on chemical composition of avocado essential oil

3.3 Antimicrobial activity

When tested against a panel of seven microorganisms, essential oils from all cultivars did not exhibit any significant fungicidal or bactericidal activity against either *Aspergillus niger*, *Candida albicans*, *Pseudomonas aeruginosa* or *Escherichia coli*. However, good bactericidal activities were observed against Gram positive bacteria (*Streptococcus mutans* and *Staphylococcus aureus*) as well as the Gram negative *Salmonella typhimurium*. Avocado essential oils showed the strongest antimicrobial activity against *S. mutans*, the Gram positive anaerobe contributing to tooth decay and other oral cavity infections. While standard drug ciprofloxacin showed an IC_{50} at 3.9 μg/mL, avocado essential oils showed IC_{50} ranged from 0.42-2.8 μl/mL justifying the folk use of avocado leaves in treatment of oral cavity infections [12]. Concentration kill curves are displayed in Figure 2.

Among the investigated essential oils, that of Bacon cultivar was the most potent towards *S. typhimurium* (IC_{50} 0.9 μl/mL versus 0.98 μg/mL for ciprofloxacin)

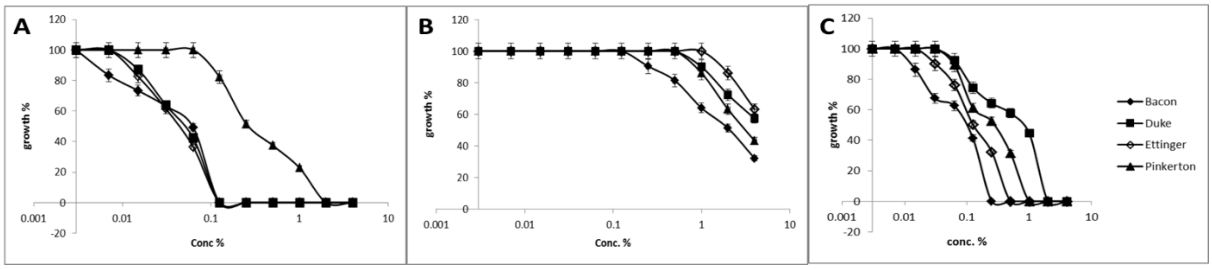

Figure 2: Concentration-kill curve of the essential oil from different avocado cultivar against A, *S. mutans*; B, *S. aureus*; C, *S. typhimurium* over concentration range between 0.03 to 40 μL/mL. Bacteria growth is expressed on the y axis based on the optical density of bacterial culture measured at 600 nm when compared with control culture.

Table 3: Average linkage between cultivars as calculated by Unweighted Pair Group Method using Arithmetic Average (UPGMA) from RAPD analysis

Cultivar	Bacon	Duke	Ettinger	Pinkerton
Bacon	100	80	95	87
Duke	80	100	78	80
Ettinger	95	78	100	86
Pinkerton	87	80	86	100

and *S. aureus* (IC50 21 µl/mL versus 1.95 µg/mL for Ciprofloxacin). This variability in efficiency may be due to either synergistic or antagonistic effect of the oxygenated (estragole and methyl eugenol) and non-oxygenated constituents (*α*-pinene and *β*-pinene) in the essential oil mixture.

4. Conclusion

In conclusion, this study represents the first comprehensive account of the chemical composition and antimicrobial activity of the essential oils from avocado cultivars introduced in Egypt. Our findings suggests that further evaluation of the medicinal value of avocado leaves should include assessment of the inherited chemotypic variation between its cultivars based on their original linage

Table 2: Essential oils' composition of four avocado cultivars as percentage values determined by GC/FID

Compound	Peak area %				
	KI	Bacon	Duke	Ettinger	Pinkerton
Cyclohexanemethane	729	t.	-	-	-
Hexanal	803	t.	-	t.	-
2-Hexanal	860	t.	-	-	-
α-Pinene	937	3.44	1.02	5.27	13.89
Camphene	954	t.	t.	t.	-
Sabinene	977	t.	t.	t.	2.96
β-Pinene	982	6.89	1.76	**13.32**	**22.04**
β-Myrcene	991	0.42	0.09	t.	1.47
α-Phellandrene	1010	-	t.	-	1.24
m-Acetylphenol	1021	t.	-	-	-
p-Acetyltoluene	1028	t.	-	-	0.35
α-Myrcene	1033	t.	t.	t.	1.4
Eucalyptol	1038	1.59	0.31	3.49	3.09
Dispiro[2.1.2.1]octane	1049	-	-	0.18	t.
Estragole	1204	**41.86**	**93.49**	**52.88**	**29.54**
Methyl eugenol	1396	**31.11**	0.75	**17.73**	13.44
β-Caryophyllene	1429	3.97	1.1	3.19	4.6
(+)-Epi-bicyclosesquiphellandrene	1439	0.41	0.11	0.29	0.63
α-Caryophyllene	1461.5	0.73	-	0.47	0.87
Dihydro-cis-α-copaene-8-ol	1516.3	0.8	0.35	0.34	0.88
Unidentified compound		8.78	1.02	2.84	3.6
Identified compounds		91.22	98.98	97.16	96.4
Monoterpene hydrocarbons		10.75	2.87	18.59	43
Oxygenated monoterpenes		1.59	0.31	3.49	3.09
Sesquiterpene hydrocarbons		5.11	1.21	3.95	6.1
Oxygenated sesquiterpenes		0.8	0.35	0.34	0.88
Aromatic compound		72.97	94.24	70.61	42.98

Conflicts of interest

There are no conflicts to declare.

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