A Comparative Pharmacognostical Study of Certain Avocado (*Persea americana* Mill.) Cultivars Grown in Egypt

A thesis presented by

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INTRODUCTION

Avocado (*Persea americana* Mill., Lauraceae) is an evergreen tree native to Central America. The plant is cultivated in both tropical and Mediterranean regions for its delicious and nutritious fruits. Avocado fruit or “Butter fruit” was described as a fleshy one-seeded drupe that contains unusual high percentage of lipids reaching up to 20% of dry weight (Fumio and Kaori, 2008). The fruit is green- or purple- to black-skinned and spherical, pear- or egg-shaped. Avocado has a good market worldwide; the largest producer and exporter of avocado fruit is Mexico (Rhoda and Burton, 2015).

Avocado fruit and its pericarp fixed oil are usually considered as efficient components of healthy antioxidant diets. In fact, the lipoidal composition of avocado oil was found to be closely similar to that of olive oil and even more enriched with unsaturated fatty acids; besides, the oil is characterized by its high carotenoid and tocopherol contents (Finau, 2011). Due to the combined effects of these antioxidant principles, avocado is chiefly recommended for people with high risk of heart diseases and patients suffering from atherosclerosis (Brai *et al*., 2007). Moreover, owing to its high phytosterol content the extract of the fruit pericarp, combined with soybean oil, is indicated as supportive treatment for arthritis being marketed in Egypt under the trade name, Piascledine® (Copad Egypt, 2015).

The importance of avocado fruit oil is further extended to cosmetic industry. In that concern, it plays a valuable role in skin-care preparations due to ease of penetration, remarkable healing and softening properties, and high vitamin (A, D and E) and sterol contents (Finau, 2011; de Oliveira *et al*., 2013).

Apart from the nutritional and economical value of its fruits, several folk medicinal uses have been reported for the different organs of *Persea americana*. In Mexico, the leaves are traditionally taken as cough remedy and externally applied to heal bruises (Anderson, 2003); while the seed powder is used in Nigeria in case of chronic hypertension (Ozolua *et al*., 2009). Both leaves and fruits are also utilized as antidiabetic (Oboh *et al.*, 2014); and leaves, seeds and bark for treatment of stomachache, dysentery and diarrhea. The fruit oil is said to be, as well, efficient for wound healing, and promoting hair growth (Morton, 1987; Dermarderosian and Beutler, 2002).

Scientific research has provided evidence supporting the traditional medicinal importance of avocado. The ethanolic extract of the dried leaves was found to exhibit an anti-inflammatory effect in carrageenan-induced edema in rats (Guevarra *et al.*, 1998) and *in-vitro* anti-oxidant activity (Rodríguez-Carpena *et al.*, 2011). Persin, isolated from the leaves, has been proven to exert a cytotoxic activity toward mammary glands, and was thus suggested as a new prototype for development of selective chemotherapeutic agents for treatment of breast cancer (Butt *et al*., 2006). While, the acetone extract of the pericarp was shown to inhibit the proliferation of human prostate cancer cell line (Lu *et al*., 2005). In addition, the anti-hypertensive activity of the aqueous extract of the seed has been confirmed by a number of studies (Anaka *et al.,* 2009; Yasir *et al.,* 2010). Furthermore, there is a plethora of scientific evidence on the antibacterial (Lu *et al*., 2012), anticholesterolemic, hypoglycemic and antihypertensive activity (Yasir *et al,* 2010) of avocado fruit and seed.

Among over 1000 avocado cultivars distributed worldwide, some have been naturalized in the Middle East during the last century (Encyclopedia Britannica, 2015). Systematically, all avocado cultivars are reported to originate from three races: the Mexican, Guatemalan and West Indian (Miller, 1754; Scora and Bergh, 1992). The Mexican and Guatemalan varieties and their hybrids were found the most successfully grown in temperate regions (Bender, 2014). Accordingly, certain cultivars have been introduced in Egypt among these are the Bacon, Duke, Ettinger, Fuerte, Gwen, Hass and Pinkerton which are all mainly propagated as fruit crops.

Based on this important economic value from both nutritional and medicinal standpoints, it was found crucial to comparatively investigate the chemical and biological differences among the aforementioned seven avocado cultivars. Furthermore, these plants are produced through a process of repeated cross hybridization thus their genetic makeup is expected to vary widely.

To fulfill these goals the following steps were performed:

1. An updated literature survey summarizing the reports concerned with the chemical composition and bioactivity of avocado (*Persea americana* Mill., Lauraceae).
2. Determination of the genetic relatedness among seven avocado cultivars naturalized in Egypt *via* DNA fingerprinting.
3. Establishment of macro- and micro-morphological criteria for inter-cultivar differentiation through examination of leaf and fruit of the seven cultivars.

4. Phytochemical and chromatographic screening of the leaves of the different cultivars.

5. Determination of the essential oil contents of the leaf samples.

6. Quantitative determination of steroids, terpenoids, phenolics and flavonoids of ethanol extracts of the leaves of the seven cultivars.

7. Investigation of the chemical composition and antimicrobial properties of the leaf essential oils of selected cultivars.

8. Determination of lipid, protein, vitamin and phenolic contents of the mesocarp of selected cultivars.

9. Qualitative and quantitative analysis of the saponifiable and unsaponifiable components of the lipoidal fraction of the fruit mesocarp of selected cultivars.

10. Evaluation of the anti-inflammatory and anti-oxidant activities of the lipoidal fraction and ethanol extract of the mesocarp of selected cultivars.

ABSTRACT

Avocado (*Persea americana* Mill., F. Lauraceae) is an evergreen tree, native to Central America and cultivated in both tropical and Mediterranean regions including Egypt. The nutritional and medicinal uses of avocado have been confirmed through numerous studies. In this respect, seven avocado cultivars grown in Egypt (one pure Mexican and six Mexican-Guatemalan hybrids) were investigated in view to provide genetic, botanical, phytochemical and biological criteria for inter-cultivar discrimination. The Duke (pure Mexican cultivar) was the most genetically distant as demonstrated by DNA fingerprinting while high similarities were observed between the remaining cultivars. Different botanical and phytochemical features were detected among leaves and fruits of the investigated samples offering suitable criteria for inter-cultivar differentiation. The investigation of the composition and antimicrobial activity of the essential oils of four of the cultivars (selected based on highest essential oil yield) revealed variability in both composition and bioactivity. A total of 20 constituents were identified with estragole, methyl eugenol and β-pinene as major. Chemometric analysis performed on the GC/FID and GC/MS data indicated close similarity between the essential oils of Bacon and Ettinger cultivars, while that of the Duke was the most distant. This classification was further supported by comparative DNA fingerprinting using RAPD technique. Essential oils of all cultivars showed good antimicrobial activities against *Streptococcus mutans* and *Salmonella typhimurium* (IC_{50} values, 0.4-20 μL/mL). The investigation of the lipoidal composition of the mesocarps of the most genetically distant cultivars (Duke and Fuerte) showed a higher concentration of unsaturated fatty acids and phytosterols in that of Duke. The mesocarp of Fuerte cultivar was found to contain higher amounts of vitamins A and C, while that of Duke was richer in total protein and vitamin E contents. The fixed oils derived from the mesocarps of both cultivars showed significant although different anti-inflammatory activities when compared to indomethacin; meanwhile, the ethanolic extract of the defatted mesocarp of Duke cultivar showed a distinctly higher anti-oxidant activity than that of Fuerte. The current study revealed that the bioactivity of both avocado leaves and fruits is obviously influenced by the kind of cultivar due to difference in composition. This necessitates that the type of cultivar implemented in any avocado-containing dietary supplement should be clearly specified.

AIM OF THE WORK

1. Provide genetic, botanical, phytochemical and biological criteria for discrimination between seven of the avocado cultivars grown in Egypt.

2. Assess how the genotypic variations among cultivars are translated into morphological and histological characteristics as well as distinct chemotypes and subsequently different medicinal value.

GENERAL SUMMARY

The plants under investigation are seven cultivars *viz.*; Bacon, Duke, Ettinger, Fuerte, Gwen, Hass and Pinkerton of *Persea americana* Mill. family Lauraceae. The latter includes 50 genera and close to 3000 species of mostly evergreen tropical or subtropical trees and shrubs with leathery leaves. Genus *Persea* (Mill.) comprises about 150 species grouped into 2 subgenera: subgenus *Persea* which includes *Persea americana* and subgenus *Eriodaphne*.

Avocado fruit and fixed oil of the mesocarp are recommended in healthy antioxidant and anti-inflammatory diets. Moreover, many researches have proven that the lipoidal composition of avocado oil closely resembles that of olive oil and is even more enriched with unsaturated fatty acids. In addition, avocado oil contains a higher carotenoid and tocopherol contents.

Based on this important economic value from both nutritional and medicinal standpoints, it was found crucial to comparatively investigate the chemical and biological differences among the aforementioned seven avocado cultivars. Furthermore, these plants being produced through a process of repeated cross hybridization are expected to show widely variable genetic makeup. Therefore, the present study was conducted in view to:

1. Provide genetic, botanical, phytochemical and biological criteria for discrimination between seven of the avocado cultivars growing in Egypt.
2. Assess how the genotypic variations among cultivars are translated into morphological and histological characteristics as well as distinct chemotypes and subsequently different medicinal value.

Part I: Genetic, Botanical and Chemical Profiling

Chapter I: Genetic Profiling

Genetic profiling using RAPD analysis with ten different decamers provided helpful criteria for the discrimination between the seven avocado cultivars under investigation. Moreover, the correlation between genetic similarity and the horticultural race(s) that each cultivar originates from was proven by this analysis.

The highest similarity between the investigated avocado cultivars was found to be 95% between Bacon and Ettinger followed by Hass and Pinkerton cultivars with 89% genetic similarity. Duke cultivar (the only pure Mexican cultivar included in this study) was the most genetically distant from all other cultivars.

Chapter II: Botanical Profiling

Macro- and micro-morphological examination of the leaves and fruits of the seven investigated avocado cultivars provided further criteria that can be used for inter-cultivar differentiation.

1. Variability in size and odor among leaf laminae can be used to differentiate between different cultivars. Leaf of Duke cultivar is considered as the smallest leaf while only the leaves of Bacon, Duke, Ettinger and Pinkerton have a characteristic odor.
2. Cross sections in the leaf laminae showed similar features except for the lignified pericyclic fibers in the midrib region which was completely absent or rarely present in Duke cultivar. Moreover, the midrib prominence to the upper surface was not observed in Duke and Ettinger cultivars.

3. General characteristics of both the upper and lower epidermal cells, especially the recorded numerical values, appeared to be most decisive in differentiating between the seven cultivars.

- For example, Fuerte and Hass cultivars could be distinguished from the others by the absence of covering trichomes, and from each other by the stomatal values which are noticeably higher in the Hass (stomatal number and index; 536 and 39, respectively).
- Among the five hairy cultivars, the Bacon could be recognized *via* its distinctly lower stomatal values (272 and 29, for stomatal number and index, respectively). Meanwhile, the four others (Duke, Ettinger, Gwen and Pinkerton) of close stomatal indices (33-35) could be easily differentiated by the frequency and size of the covering trichomes which appeared more abundant and longer in the lower epidermis of the Duke cultivar.

Chapter III: Chemical Profiling

I. Preliminary phytochemical screening

Alkaloids and anthraquinones were absent from all investigated cultivars. Meanwhile, all the investigated cultivars were found to contain volatiles, steroids, triterpenoids, flavonoids, saponins and tannins as common constituents.

II. Chromatographic screening and fingerprinting

Screening for phenolics using TLC resulted in the detection of 10 spots. The highest number of spots (10) was found in Duke cultivar, while the least number (4) was found in Fuerte cultivar. Moreover, two spots with R_f of 0.32 and 0.93 were identified as rutin and gallic acid by co-chromatography, respectively. Screening using HPLC resulted in the detection of 4 peaks that were common in all the samples. Two peaks with retention time at 2.625 and 10.317 minutes were identified as gallic acid and rutin, respectively, through comparison with authentic samples run under the same experimental conditions thus confirming the results obtained by TLC.

III. Quantitative determination of selected secondary metabolites

A. Determination of volatiles

Hydrodistillation was carried out in a Clavenger-type apparatus on 100 g leaf samples. Percentage yields v/w (calculated on dry weight basis) were found to be:

- Duke = 1%
- Ettinger and Bacon = 0.4%
- Pinkerton = 0.25%
- Gwen and Hass = 0.1%
- Fuerte = 0.05%

B. Determination of steroid and terpenoid content

Colorimetric determination of these constituents was carried out by the vanillin/H_2SO_4 method. Ettinger leaf sample was found to be the most enriched with the steroids and/or terpenoids with a concentration of 391.37 mg ursolic acid equivalent/g followed by those of Pinkerton and Duke cultivars by a concentration of 380.25 and 332.48 mg ursolic acid equivalent/g of dried leaves, respectively.

C. Determination of phenolics

Total phenolics were determined in the leaf samples by using Folin-Ciocalteu reagent and expressed as mg gallic acid equivalent/g dry plant. Flavonoids were estimated as mg quercetin equivalent/g dry plant by means of the $AlCl_3$ method. The Duke cultivar was found to be the most enriched in phenolics followed by Hass and Bacon. On the other hand, the flavonoid content was detected in highest amount in Hass followed by Duke then Ettinger.

Concentrations of rutin and gallic acid, as determined by HPLC on ODS-3 C18 column (particle size 5 µm, 250mm × 4.6 mm∅) and isocratic elution, varied widely among different avocado cultivars. Pinkerton cultivar contained the highest amount of rutin followed by Duke then Ettinger. On the other hand, Duke cultivar contained the highest amount of gallic acid followed by both of Ettinger and Pinkerton cultivars.

Part II: Investigation of the Essential Oils of the Leaves

The leaves of Bacon, Duke, Ettinger and Pinkerton cultivars with highest yeilds of essential oils were selected for this study.

Chapter I: Investigation of the Chemical Composition

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 composition from Bacon, Duke, Ettinger, and Pinkerton cultivars, respectively. All essential oils were enriched in aromatic compounds, namely estragole and methyl eugenol.

Estragole was the single major constituent (present at 93.5%) in the essential oil from the pure Mexican cultivar, Duke. Estragole prevalence was found in different ratios in the essential oils under investigation. In addition, the decrease in estragole percentage obviously correlated with the Mexican component of hybrid cultivars. In fact, among the analyzed samples, Pinkerton cultivar (the predominately Guatemalan hybrid) showed the lowest estragole content (29.5%) and the highest ratios of hydrocarbons represented principally by α-pinene and β-pinene.

Both hierarchal cluster analysis (HCA) and principle component analysis (PCA) of the chemical composition of the four essential oils resulted in the grouping of the two predominantly Mexican cultivars (Bacon and Ettinger), while, the pure Mexican cultivar, Duke was the most distinct. This result came in total agreement with the genetic profiling performed using RAPD analysis.

Chapter II: Evaluation of the Anti-Microbial Activity

Essential oils from the four cultivars did not exhibit any significant fungicidal activity against either *Aspergillus niger* or *Candida albicans.* However, good bactericidal activities were observed against Gram positive bacteria (*Streptococcus mutans* and *Staphylococcus aureus*) as well as the Gram negative *Salmonella typhimurium*. No bactericidal activity was observed against *Pseudomonas aeruginosa* and *Escherichia coli* at the concentration used in the study.

Bacon cultivar was found to be the most active with the lowest MIC and IC_{50} against *S. mutans, S. aureus* and *S. typhimurium.* This activity may be attributed, at least partially, to the highest methyl eugenol content when compared to other investigated oils. The antimicrobial activity observed for the other cultivars seemed also to correlate with their methyl eugenol content.

Part III: Investigation of the Mesocarps of the Fruits

The fruits of the two most genetically distant cultivars *viz.*; Duke and Fuerte were selected for this investigation.

Chapter I: Determination of Lipid, Protein, Vitamin and Phenolic contents

The mesocarp of fruit of Fuerte cultivar was found to contain a higher amount of vitamins A (18.03 ppm) and C (42.5 ppm) and fixed oil (52 mL/g). While, mesocarp of the fruit of Duke cultivar was found to contain a higher amount of total protein (10.2 g %) and vitamin E (40 ppm).

The ethanolic extract of the defatted mesocarp of Duke cultivar was found to contain a higher amount of phenolics than that of the Fuerte.

Chapter II: GLC Profiling of the Unsaponifiable and Saponifiable Lipoids:

A qualitative and quantitative variability was evident among lipoidal constituents of the mesocarps of the two cultivars. The unsaponifiable fraction of the fixed oil of Duke cultivar was found more enriched in steroids while hydrocarbons predominated in that of Fuerte.

Unsaturated FA prevailed in the saponifiable fraction of both oils being higher in the Duke; while saturated FA occurred in higher percentage in the Fuerte cultivar. Oleic acid constituted about 50% of FA components in both cultivars.

Chapter III: Evaluation of the Biological Activity of the Mesocarp

I. Anti-inflammatory activity of the fixed oil

Fixed oils of both the Duke and Fuerte cultivars showed a significant anti-inflammatory activity at 15 mg/kg b.wt. dose when tested by the rat paw oedema method. The anti-inflammatory effect of avocado fixed oil was comparable to that of the non-steroidal anti-inflammatory drug indomethacin at a dose of 10 mg/kg b.wt. There was no significant difference between the anti-inflammatory activities of the Duke and Fuerte fixed oils at 15 mg/kg b.wt., while that of the Fuerte cultivar showed significantly higher activity at the lower dose of 7.5 mg/kg b.wt.

II. Anti-oxidant activity of ethanol extract of the defatted mesocarp.

Both of Duke and Fuerte extracts showed DPPH-radical scavenging activities with the Duke extract showing much stronger effect that was comparable to ascorbic acid (EC_{50} of Duke 27.21 µg/mL *vs.* EC_{50} of ascorbic acid 23.53 µg/mL). This difference in anti-oxidant activities between both cultivars may be attributed to the higher concentrations of vitamin E and phenolics recorded in the Duke mesocarp.

This study confirms the suitability for application of avocado fruit pulp (mesocarp) as anti-inflammatory and anti-oxidant especially that of the Duke cultivar in phytopharmaceutical preparations.

Conclusion and Recommendations

- Avocado is an economically valuable edible fruit crop with many established health benefits.
- Avocado cultivars introduced in Egypt showed considerable genetic variability that is translated into distinct morphological features and chemotypic characteristics.
- Certain cultivars (e.g. Duke, locally known as Egyptian avocado) could be readily distinguished from the others by the morphological characters of its leaves and fruits.
- Measurement of certain numerical values such as stomatal and hair numbers per mm^2 were found useful in identify avocado cultivars that could not be distinguished based solely on morphological features.
- Avocado leaves and fruits showed almost similar qualitative chemical profiles; yet, these were distinctly different quantitatively.
- Variations among leaves metabolites provided reliable criteria for inter-cultivar differentiation even before fruiting.
- The essential oils of avocado leaves (an agricultural byproduct) showed good antibacterial activity and seemed especially useful in oral cavity infections.
- Interestingly, the fruit of Duke cultivar showed better health-promoting lipid profile (higher phytosterol and vitamin E contents) when compared to the globally prevalent Fuerte. Thus, it is strongly recommended for further agricultural expansion.
- The study revealed that the medicinal value (anti-inflammatory and anti-oxidant) of both avocado leaves and fruits is strongly dependent on the type of cultivar. This implies that the type of cultivar should be clearly specified in any avocado-containing dietary supplement.