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DNA Fingerprinting, Chemical Composition, Antitumor and Antimicrobial Activities of the Essential Oils and Extractives of four Annona Species from Egypt

Seham S. Elhawary[1], Mona E. El Tantawy[2], Mohamed A. Rabeh[1,*] and Noha E. Fawaz[2]
1. Pharmacognosy department, faculty of Pharmacy, Cairo University, Cairo, Egypt.
2. Tissue culture department, National Organization for drug control and research (NODCAR), Giza, Egypt.
*Corresponding author: E-mail: mohamedabdelatty68@yahoo.com

Abstract

The leaf essential oils of four members of the Annonaceae grown in Egypt (namely; *Annona cherimola, A. squamosa, A. muricata and A. glabra*) have been obtained by hydrodistillation and analyzed by GC-MS in order to compare and contrast the volatile chemical compositions of these species. The essential oils were screened for in-vitro cytotoxic activity against breast cancer (MCF-7), colon cancer (CACO-2) and liver cancer (HEPG2) cell lines and antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeuroginosa, Aspergillus flavus* and *Candida albicans*. β-elemene (25.02%), β-caryophyllene (37.11%), bicycloelemene (23.58%) and β-gurjunene (42.49%), were the major constituents of *Annona cherimola, A. squamosa, A. muricata and A. glabra,* respectively. Ethanol extracts showed highly significant cytotoxic activities (low IC50) much more than results displayed by essential oils on breast (MCF-7), colon (CACO-2) and liver (HEPG2) carcinoma cell lines. Relative to breast carcinoma cell line (MCF-7), The IC50 values of ethanol extracts were 3.43, 3.89 and 4.34 µg/ml for *A. cherimola, A. squamosa* and *A. muricata* ethanol extractives respectively. While colon carcinoma cell line (CACO-2) displayed IC50 values 2.82, 2.97, 3.58 and 3.89 µg/ml for *A. muricata, A. cherimola, A. glabra* and *A. squamosa*, respectively. Liver carcinoma cell line (HEPG2) exhibited IC50 values of 3.12, 3.43 and 3.73 for *A. squamosa, A. muricata* and *A. cherimola*, respectively. Three of the four leaf essential oils showed notable *invitro* cytotoxic activity. Essential oils of *A. glabra, A. muricata* and *A. squamosa* showed moderate cytotoxic activities with IC50 values ranging from 12.35 to 24.21µg/ml. While the essential oils of *A. cherimola* showed IC50 values ranging from 7.67 to 9.22µg/ml. Leaf oils and ethanol extractives showed appreciable antibmicobial activity with variable MIC ranging from 30 to 315µg/ml. These findings suggest that *A. cherimola* essential oil and ethanol extract have great potential as a natural medicine for cancers and microbial infections.

Keywords: *Annona cherimola; A. squamosa;, A. muricata ; A. glabra*; Annonaceae; DNA, essential oil composition; cytotoxicity; antimicobial

1. Introduction

Annonaceae family, also called custard apple family, is a family of flowering plants consisting of trees and shrubs. It is one of the largest and most diverse plant families, composed of around 2300 to 2500 species and more than 130 genera; it is the largest family in the Magnoliales. Only four genera, *Annona, Rollinia, Uvaria* and *Asimina* produce an edible fruit which is known as "anona". The family is concentrated in the tropics, with few species found in temperate regions. Annonaceous plants are cultivated as a source for the edible fruits (Mabberley, 1997).

Leaves, bark and roots of some *Annona* species are used in folk medicines. Pharmaceutical research has found antifungal, bacteriostatic, and especially cytostatic capability of some chemical constituents of the leaves and bark. A large number of chemical compounds, including flavonoids, alkaloids and acetogenins, have been extracted from the seeds and many other parts of these plants (Gajalakshmi *et al*. 2012; Pandey and Barve 2011). Flavonoids and alkaloids have shown antibacterial properties, and have been used for treatment of medical conditions, such as skin disease, intestinal worms and inflammation of the eye (Pandey and Barve 2011).

Acetogenins are anticipated to have anti-HIV and anti-cancer effects. Wide varieties of products have been developed and are available for cancer treatment. Flavonoids and alkaloids content in the leaves and bark of several species of the family have shown insecticidal properties (Chen, *et al*. 2012).

Many members of the Annonaceae have been characterized in terms of volatile oil analysis (Ekundayo, 1989). Some members of the family are important in local traditional medicine. However, previous work focused in just a few members of this large (over 25000 species) plant family. To date, not all of the species have been soundly studied seeking potential bioactive components. Little has been done about the antitumor, antimicrobial activities of *Annona* species leaves essential oils and extractives. This study aimed to investigate the chemical composition of four *Annona* species, collected from Cairo, Egypt; essential oils by GC/MS. Additionally the study assessed the antitumor and antimicrobial properties of essential oils and extracts from leaves.

2. Materials and Methods

2.1. Plant Material

Samples of four *Annona* species (*A. cherimola M., A. glabra L., A. muricata L.* and *A. squamosa* L.) were collected during the flowering season in May to July from trees growing in institute of horticultural research except *A. muricata* trees growing in Giza zoo. The plants were authenticated by Dr. Mohammed El-Gibali; senior botanist and Mrs. Terris Labib; head specialist for plant identification at El-Orman botanical garden, Giza, Egypt. Voucher specimens have been deposited in the herbarium of the El-Orman botanical garden. Samples of the leaves of the four Annona species under investigation were separately air-dried in the shade, powdered and kept in tightly closed amber colored glass containers.

2.2. DNA Fingerprinting

2.2.1. Material for DNA

Fresh leaf samples were collected separately from the four Annona species; *A. cherimola* Mill., *A. glabra* Linn., *A. muricata* Linn. and *A. squamosa* Linn. All the selected leaf samples were normal and free from any pathogenic symptoms and all the samples are saved in ice box and quickly transported to laboratory. Plant tissues were ground under liquid nitrogen to a fine powder, then bulked DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN).

2.2.2. DNA extraction:

DNA was extracted separately from the leaves of *A. cherimola* Mill., *A. glabra* Linn., *A. muricata* Linn. and *A. squamosa* Linn. using the DNA extraction method described by Williams *et al.* (1990).

2.2.3. Polymerase Chain Reaction (PCR):

PCR amplification was performed using 5 random 10-mer arbitrary primers synthesized by (Operon biotechnologies, Inc. Germany). Names and sequences of the primers are shown table 1.

Table 1: List of the primer names and their nucleotide sequences used in the study

No.	Name	Sequence
1	OP-A12	5' TCGGCCATAG 3'
2	OP-C09	5' CTCACCGTCC 3'
3	OP-C13	5' CTCACCGTCC 3'
4	OP-D07	5' CATCCGTGCT 3'
5	OP-L12	5' GGGCGGTACT 3'

Amplification was conducted in 25µl reaction volume containing the following reagents: 2.5µl of dNTPs (2.5mM), 2.5µl $MgCl_2$ (2.5mM), 2.5µl of 10 x buffer, 3.0 µl of primer (10 pmol), 3.0µl of template DNA (25 ng/µl), 1µl of *Taq* polymerase (10/µl) and 10.5µl of sterile double distilled H_2O. The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. The reaction was finally stored at 72°C for 10 min.

2.2.4. Gel Electrophoresis and Staining:

Amplified products were size-fractioned using ladder marker (100 bp) Fermentas.co by electrophoresis in 1.5 % agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV florescence and photographed.

2.3. Extraction of Essential Oils

The air-dried leaves (500 g for each species) of the plants were pulverized and subjected to hydrodistillation for 5 h, using a Clevenger-type apparatus. Before analysis and biological activity test, the collected oils were dehydrated with anhydrous Na_2SO_4 and preserved at 4°C (Egyptian Pharmacopeia; 2005).

2.4. Preparation of Crude Extracts

The air-dried leaves (100 g) of each of the four *Annona* species (*A. cherimola, A. glabra, A. muricata* and *A. squamosa*) were pulverized separately. The dried powder (100 g) was extracted three times with ethanol (3 × 500 ml) at room temperature and mixed the extracts, and then it was evaporated by a vacuum rotary evaporator (Büchi R-210, Switzerland).

2.5. Gas Chromatography-Mass Spectrometry

The leaf oils of the *Annona* species were subjected to gas chromatographic-mass spectral analysis on an Agilent system consisting of a model 6890 gas chromatograph, equipped with a model 5973 mass selective detector (EIMS, electron energy, 70 eV), and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a DB-5 (5% phenyl methyl polysiloxane) stationary phase, film thickness of 0.25 µm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a flow rate of 1.0 ml/min. Inlet temperature was 200°C and MSD detector temperature was 270°C. The mass spectrometer was operated in electron impact ionization (EI) mode with 70eV energy. The mass range was 50-700 Da and the ion source temperature was 200 °C. The GC oven temperature program was used as follows: 80°C initial temperature, for 2 min.; then programmed at 15°C/min to 270°C and held for 10 min. Each sample was dissolved in acetone to give a 1% w/v solution; 1 µL injections using a splitless injection technique were used.

Identification of oil components was achieved based on their retention indices (RI, determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Rios, *et al.*, 2003; Pelissier, *et al.*, 1993) and stored on the MS libraries [NIST 05; Mass Finder database (G1036A, revision D.01.00 and Wiley7 Mass Finder]. The chemical compositions of the essential oils are compiled in Table 4.

2.6. Cytotoxicity screening

2.6. 1. Drugs and chemicals

Doxorubicin hydrochloride (DOX), as a standard cytotoxic drug, was purchased from Sigma Aldrich (St. Louis, Mo, USA). The stock solution was dissolved in phosphate buffered saline (PBS) and preserved at -20°C. The solution was diluted in Dullbecco's modified Eagles medium (DMEM) or PBS immediately before each experiment to the desired final concentrations. Dullbecco's modified eagles medium (DMEM), Phosphate buffered saline (PBS), Penicillin G and Steptomycin antibiotics, Acetic acid, Sulphorhodamine B (SRB), were purchased from Sigma Aldrich Co.

2.6. 2. Cells and cell cultures

Human adenocarcinoma cell lines; breast adenocarcinoma cell line (MCF-7), colon adenocarcinoma cell line (CACO-2) and liver adenocarcinoma cell line (HEPG2) were used in this study. They were obtained from National Cancer Institute, Cairo University, Egypt.

The adherent cells were grown as "monolayer culture" in DMEM supplemented with Penicillin (100 IU/ml), Streptomycin (100 µg/ml) and 10% Fetal bovine serum. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and were passaged every 4-5 days.

2.6. 3. Assessment of cytotoxic activity

The potential cytotoxicity of essential oils as well as ethanol extracts of the leaves of the four annona species was determined using (sulforhodamine) SRB method as previously described by Skehan *et al*., (1990) with slight modificatiions against different human adenocarcinoma cell lines; breast adenocarcinoma cell line (MCF-7), colon adenocarcinoma cell line (CACO-2) and liver adenocarcinoma cell line (HEPG-2).

Doxirubcin (DOX), the standard cytotoxic drug, was prepared as a 10 mM (6.8 mg/ml) stock solution in double-distilled H_2O and then diluted to 10 µM and stored in aliquots at -20°C, which retained activity for at least 3 months. Aqueous solution of (DOX) was filter-sterilized immediately after preparation by passage through a 0.22 µM syringe filter (Pall Corporation, East Hills, NY). Except for the initial weighing of chemicals, all solutions used for cell-culture studies were prepared under sterile conditions.

Cells were seeded in 96 well microtiter plates at a concentration of (10^4cells/well) in DMEM supplemented medium. After 24 h, cells were incubated for additional 48 h at 37°C and in atmosphere of 5% CO_2 in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, 15mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35 with various concentrations of DOX in the following ranges: 0.05-5µg/ml and essential oils and ethanol extracts (dissolved in 1% DMSO) concentrations ranged from 0.05-50µg/ml. Cells were fixed in situ by adding 50µl of cold 50% TCA for 1 h at 4°C. The supernatant is then discarded, and the wells were washed five times with distilled water, air dried, stained for 30 min at room temperature with 0.4% Sulforhodamine B stain dissolved in 1% acetic acid and then excess stain was washed four times with 1% acetic acid. The plates were air dried and the dye was solubilized with 100 µl/well of 10 mM Tris-EDTA buffer (PH 10.5) for 10 min. The optical density (OD) was obtained using ELx808 Absorbance Microplate Reader obtained from BioTek Instruments, Inc (Winooski, VT, U.S.A.) at λ_{max} 565 nm.

Surviving fraction= Optical density of treated cells/Optical density of untreated control cells

IC50 (the concentration of DOX, essential oil, and ethanol extract necessary to produce 50% inhibition of cell growth) was calculated from linear equation of the survival fraction curve.

$$Y = m X + b$$

Where: Y = 0.5 (the surviving fraction when there is a 50% inhibition of cell growth).

m = the slope.

X = dose of DOX, essential oil, and ethanol extract induces 50% inhibition.

b = the y-intercept.

The experiments were done in triplicate and the results were given as mean of the three readings. The experiment surviving fractions of the control cells (cells without any of the tested essential oil or extract) were found to be 1 for all of the three cell lines. Cytotoxic activities of the essential oils and extracts are summarized in Table 5.

2.7. Antimicrobial Screening

Antimicrobial activity of the essential oils and different extracts of the four *Annona* species was screened using Kirby-Bauer disc diffusion method (1966) with slight modification. 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media (Mueller-Hinton agar plates (HiMedia) for bacteria and Potato Dextrose Agar plates for fungi (HiMedia)) until they reached a count of approximately 10^8cells/ml for bacteria and 10^5cells/ml for fungi. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method.

Plates inoculated with filamentous fungi as *Aspergillus flavus* Link (ATCC 204304) at 25°C for 48-72 hours; Gram-positive bacteria as *Staphylococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051); Gram-negative bacteria *Escherichia coli* (ATCC 11775) and *Pseudomonas aeuroginosa* (ATCC 10145) they were incubated at 35-37° C for 24-48 hours and yeast as *Candida albicans* (ATCC 26555) incubated at 30°C for 24-48 hours. Then the diameters of the inhibition zones were measured in millimeters.

DMSO with a concentration up to 2% was used to dissolve the essential oils and plant extracts. Filter paper discs (6 mm in diameter) saturated with 20µL of the tested essential oils and extracts or DMSO (solvent control) were placed on the surface of the inoculated plates. To evaluate the efficiency of the methodology; 50µL of each essential oil was inserted simultaneously in a hole made in new plates. The plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone was measured in millimeter with slipping calipers of the National Committee for Clinical Laboratory Standards., and was recorded as mean ± SD of a triplicate experiment. Standard discs of Gentamycin (10µg, Oxoid, UK**,** Antibacterial agent), Amphotericin B (5µg, Sigma Chemical Co., St. Louis, Mo., Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 µl of solvent (DMSO) were used as a negative control. Results were shown in table 6.

Determination of the Minimal Inhibitory Concentration **(MIC)** was carried out by a serial broth dilution method described by (NCCLS, 1993) and Sahm and Washington (1991). Briefly; The essential oils and extracts were diluted in DMSO and were added to 5 ml sterile MHB tubes to give different concentrations (1.0 - 50.0 µL/ml). Later, 0.5 ml of the exponentially growing microbial broth culture of the strains that were sensitive by disc diffusion test was inoculated into respective test tubes. Another set of tubes containing only the growth medium without DMSO (control) and with DMSO (solvent control) and each of the test strains was set up separately. In 96-well plates organisms, at a concentration of approximately 1.5 x 10^8 colony forming units (CFU)/ml, were added to each well. The tubes were incubated at 37°C for 24 h and the growth was measured by measuring optical density at 520 nm using spectrophotometer comparing the sample readout with the non inoculated nutrient broth. The MIC was regarded as the lowest concentration (without turbidity) of the essential oil or extract that inhibited the growth of bacteria or fungi.

DMSO to 2% was used to dissolve the essential oil and extracts in the culture media when necessary. The control was the solvent used for essential oil and extracts and it showed no inhibitions in preliminary studies. DMSO to 2% was used to dissolve the essential oil and extracts when necessary and as a negative control whereas Gentamicin was used as a positive control for bacterial strains, Amphotericin B was used as a positive control for fungi. The plates were done in triplicate. Antimicrobial MIC results are listed in Table 7.

Statistical analysis

Statistical analysis was performed using SPSS (statistical package of social sciences, version 16). Statistical significance was acceptable to a level of $p < 0.05$.

3. Results and Discussion

3.1. DNA fingerprinting

The banding profile produced by five decamer primers (OP-A12, OP-C09, OP-C13, OP-D07 and OP-L12) used in the RAPD analysis of the four *Annona* species (*Annona cherimola* Mill., *A. glabra* Linn., *A. muricata* Linn. and *A. squamosa* Linn.) is illustrated in figure 1.

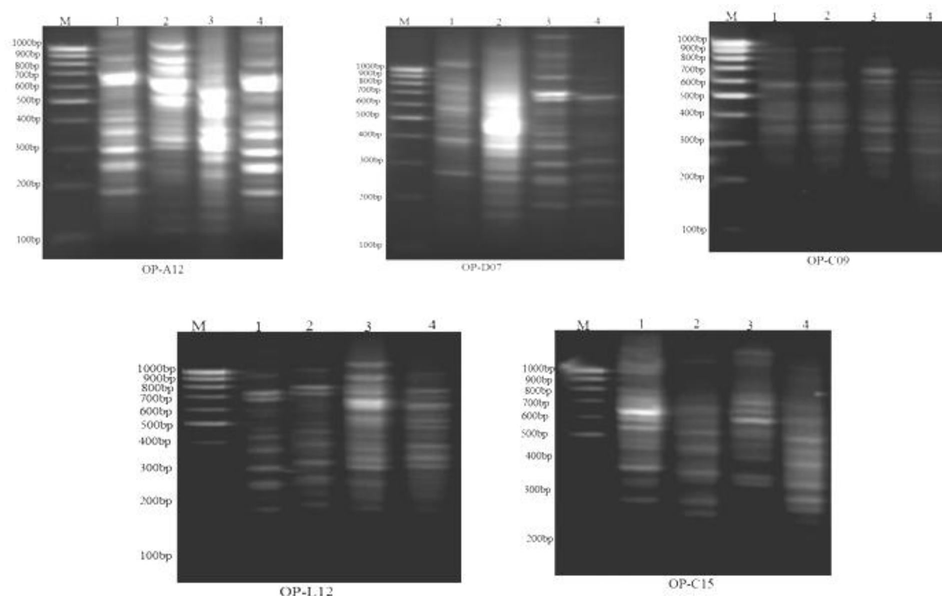

Figure 1: RAPD-PCR analysis of the four *Annona* species [*Annona cherimola* Mill.1, *A. glabra* Linn.2, *A. muricata* Linn.3 and *A. squamosa* Linn.4]

The amplified fragments and specific markers for the four *Annona* species based on RAPD-PCR analysis using five primers is shown in table 2 and the percentage of polymorphic fragments and similarity coefficient is shown in table 3.

Table 2: RAPD-PCR analysis of the four annona species [*Annona cherimola* Mill., *A. glabra* Linn., *A. muricata* Linn. and *A. squamosa* Linn.]

Primer	TAF	PB	*A. cherimola*		*A. glabra*		*A. muricata*		*A. squamosa*		TSM
			AF	SM	AF	SM	AF	SM	AF	SM	
OP-A12	18	9	13	2(-)1(+)	14	1(-)	17	-	13	1(-)	5
OP-C09	10	3	9	-	9	-	10	-	8	2(-)	2
OP-C15	20	9	17	1(+)1(-)	16	2(-)	17	1(-)	16	2(-)1(+)	8
OP-D07	21	11	12	2(-)	18	2(+)	16	1(-)2(+)	15	-	7
OP-L12	21	13	14	2(-)	17	2(-)2(+)	17	1(-)	15	2(-)	9

TAF: Total Amplified Fragments, PB: Polymorphic bands, AF: Amplified fragments, SM: Specific marker, TSM: Total Specific Markers, (+): positive marker, (-): negative marker

Table 3: % polymorphic fragments and Similarity coefficient of RAPD-PCR analysis of the four *Annona* species [*Annona cherimola* Mill., *A. glabra* Linn., *A. muricata* Linn. and *A. squamosa* Linn.]

Primer Codes	% polymorphic fragments	Similarity coefficient
OP-A12	50	50
OP-C09	30	70
OP-C15	45	55
OP-D07	52.4	47.6
OP-L12	61.9	38.1

The four plants were subjected to RAPD assay of their genomic DNA; this was performed using five different primers. The number of RAPD-PCR fragments indicates that the five were reproduced. In this study, the presence of same bands in DNA of different *Annona* species indicates degree of taxonomical relationship; also the presence of characteristic bands in DNA of each plant may help for differentiation between these plants. The **OP-L12** primer was found to be the most effective in generating polymorphic bands on application of RAPD technique as compared to the total number of RAPD fragments it generates high level of polymorphism (61.9%).

From the previous findings, it can be concluded that **OP-L12** primer can be used to discriminate between the four annona species depending on their low values of similarity coefficient and high level of polymorphism. However, the other estimated RAPD-primers, which produce high values of similarity coefficient and low levels of polymorphism, could be used in the identification of these plants.

3.2. Identification of Essential Oils

From the hydrodistillation, clear to light yellow essential oils were obtained. The percentage yield was (0.17%, 0.03%, 0.11% and 0.15% from *A. cherimola, A. glabra, A. muricata,* and *A. squamosa* respectively. A total of sixty-eight compounds were identified in the leaf oils, accounting for 85.12 - 97.74% of the total compositions of the essential oils. The chemical compositions of the leaf oils are compiled in Table 4.

The leaf essential oils of *A. cherimola, A. glabra, A. muricata,* and *A. squamosa* were made up largely of sesquiterpenes (87.57%, 86.16%, 42.29% and 81.7%, respectively), while monoterpenes were (2.54%, 7.54%, 55.05%, and 1.60%, respectively). Sesquiterpenes were major in all species except for *A. muricata* that has higher percentage of monoterpenes (55.05%). The most abundant components of the essential oil of *A. cherimola* were β-elemene (25.02%), germacrene-D (17.71%), and β-caryophyllene (9.50%), while *A. glabra* was dominated by β-caryophyllene (37.11%), γ-muurolene (19.19%) and α-humulene (11.12%) whereas *A. muricata* leaf oil was also characterized by abundant monoterpenes (55.05%), chiefly limonene (16.85%) and β- pinene (14.30%) while *A. muricata* sesquiterpenoids (42.29%) contained principally bicycloelemene (23.58%). *A. squamosa* essential oil was dominated by sesquiterpenes; β-gurjunene (42.49%), viridiflorene (6.68%) and γ-muurolene (5.72%). Non-oxygenated compounds were of higher percentages (82.53%, 82.68%, 85.82%, and 73.69%) while oxygenated ones were (8.39%, 12.05%, 11.92% and 11.43%) respectively.

Table 4: Identified constituents and the relative percentage of volatile oil constituents in the four *Annona* species.

No			Mass spectra			Area%			
	Name[a]	**KI**[b]	**MW**	**B**	**m/z**	***A. c.***	***A. g.***	***A. m.***	***A. s.***
1	α-Pinene	**939**	**136**	**93**	77, 41, 53, 121, 67	**-**	**-**	**5.43**	**-**
2	α-Fenchene	**952**	**136**	**93**	79, 41, 121, 107, 67	**-**	**-**	**6.58**	**1.14**
3	Camphene	**954**	**136**	**93**	121, 41, 79, 67, 107	**-**	**-**	**-**	**0.29**
4	β-Pinene	**979**	**136**	**93**	41, 69, 79, 53, 121	**-**	**-**	**14.30**	**-**
5	δ-2-Carene	**1002**	**136**	**93**	121, 77, 41, 136, 105	**-**	**2.22**	**-**	**-**
6	δ-3-Carene	**1011**	**136**	**93**	77, 41, 121, 136, 105	**-**	**5.32**	**-**	**-**
7	α-Terpinene	**1017**	**136**	**121**	93, 136, 77, 105, 43	**-**	**-**	**2.63**	**-**
8	Limonene	**1029**	**136**	**68**	93, 79, 53, 41, 121	**0.23**	**-**	**16.85**	**-**
9	(E)- β-Ocimene	**1050**	**136**	**93**	79, 41, 105, 121, 53	**2.02**	**-**	**3.25**	**-**
10	γ-Terpinene	**1059**	**136**	**93**	77, 136, 121, 43, 105	**-**	**-**	**2.04**	**-**
11	Terpinolene	**1088**	**136**	**93**	121, 136, 79, 43, 105	**0.29**	**-**	**1.79**	**-**
12	n-Nonanal	**1100**	**142**	**41**	57, 70, 82, 98, 114	**-**	**-**	**0.74**	**-**
13	Menthone	**1152**	**154**	**112**	41, 69, 55, 139, 97	**-**	**-**	**0.79**	**-**
14	Terpinen-4-ol	**1177**	**154**	**71**	43, 93, 111, 55, 86	**-**	**-**	**0.12**	**-**
15	(2E)-Hexenyl isovalerate	**1247**	**184**	**57**	85, 41, 67, 142, 155	**-**	**-**	**0.53**	**-**
16	Bornyl acetate	**1285**	**196**	**95**	43, 136, 121, 80, 108	**-**	**-**	**-**	**0.17**
17	Bicycloelemene	**1330**	**204**	**121**	93, 41, 107, 79, 136	**-**	**-**	**23.58**	**-**
18	δ-Elemene	**1338**	**204**	**121**	93, 136, 41, 161, 77	**4.46**	**-**	**-**	**0.66**
19	α-Cubebene	**1351**	**204**	**105**	161, 119, 91, 41, 204	**-**	**-**	**0.34**	**-**
20	α-Copaene	**1376**	**204**	**161**	119, 105, 91, 41, 81	**0.88**	**0.33**	**0.16**	**-**
21	β-Patchulene	**1381**	**204**	**189**	161, 119, 105,204,41	**1.12**	**-**	**-**	**-**
22	β-Elemene	**1390**	**204**	**93**	81, 41, 67, 107, 53	**25.02**	**-**	**2.04**	**-**
23	Z-caryophyllene	**1408**	**204**	**41**	93, 79, 69, 133, 105	**0.16**	**-**	**-**	**0.65**
24	α-Gurjunene	**1409**	**204**	**204**	161, 189, 105,119,91	**-**	**-**	**0.22**	**-**
25	β-Caryophyllene	**1419**	**204**	**41**	93, 133, 79, 69, 105	**9.50**	**37.11**	**-**	**-**
26	β-Gurjunene	**1433**	**204**	**161**	105, 119, 91, 41, 189	**-**	**-**	**3.12**	**42.49**
27	γ-Elemene	**1436**	**204**	**121**	93, 41, 107, 67, 79	**3.50**	**-**	**-**	**-**
28	(Z)- β-Farnesene	**1440**	**204**	**41**	69, 93, 133, 79, 55	**-**	**-**	**-**	**2.35**
29	Aromadendrene	**1441**	**204**	**41**	161, 91, 105, 79, 119	**1.79**	**-**	**-**	**5.49**
30	α-Humulene	**1454**	**204**	**93**	80, 41, 121, 147, 67	**2.20**	**11.12**	**-**	**-**
31	Alloaromadendrene	**1460**	**204**	**41**	91, 105, 161, 79, 133	**-**	**-**	**-**	**0.55**
32	γ-Gurjunene	**1477**	**204**	**161**	105, 81, 91, 41, 119	**2.25**	**-**	**-**	**0.82**
33	γ-Muurolene	**1479**	**204**	**161**	105, 119, 204, 91, 41	**-**	**19.19**	**-**	**5.72**
34	Germacrene-D	**1481**	**204**	**161**	41, 105, 91, 79, 119	**17.71**	**0.71**	**1.02**	**-**
35	α-Amorphene	**1484**	**204**	**105**	41, 94, 161, 119, 77	**-**	**0.55**	**-**	**0.96**
36	β-Selinene	**1490**	**204**	**105**	204, 93, 41, 79, 189	**-**	**-**	**-**	**0.21**
37	Bicyclogermacrene	**1495**	**204**	**121**	93, 107, 41, 79, 161	**6.30**	**-**	**-**	**-**
38	Viridiflorene	**1496**	**204**	**107**	41, 93, 161, 119, 79	**-**	**-**	**-**	**6.68**
39	Valencene	**1496**	**204**	**161**	204, 91, 41, 105, 79	**0.81**	**-**	**-**	**-**
40	α-Selinene	**1498**	**204**	**189**	204, 133, 93,107,161	**-**	**-**	**0.18**	**-**
41	Epizonarene	**1501**	**204**	**161**	81, 105, 41, 119, 204	**-**	**-**	**0.54**	**3.86**
42	γ-Cadinene	**1513**	**204**	**161**	105, 91, 119, 41, 204	**0.42**	**0.54**	**0.80**	**-**
43	δ-Cadinene	**1523**	**204**	**69**	204, 119, 105,134,91	**2.38**	**3.29**	**0.63**	**-**
44	(E)- γ-Bisabolene	**1531**	**204**	**93**	107, 41, 119, 79, 135	**-**	**2.30**	**-**	**-**
45	Elemol	**1549**	**222**	**59**	93, 43, 161, 107, 67	**2.49**		**-**	**-**
46	(E)-Nerolidol	**1563**	**222**	**41**	69, 93, 107, 55, 81	**0.75**	**0.84**	**-**	**-**
47	Spathulenol	**1578**	**220**	**43**	205, 91, 119,105,159	**-**	**-**	**1.01**	**-**
48	Caryophyllene oxide	**1583**	**220**	**41**	79, 93, 69, 55, 109	**-**	**4.22**	**-**	**-**
49	Viridiflorol	**1592**	**222**	**43**	109, 161, 69, 81, 55	**-**	**-**	**0.41**	**-**
50	Epi- α-Cadinol	**1640**	**222**	**161**	204, 81, 43, 105, 119	**-**	**-**	**4.38**	**0.23**
51	Alloaromadendrene epoxide	**1641**	**220**	**41**	91, 55, 79, 67, 105	**-**	**-**	**-**	**5.31**
52	Epi- α-Muurolol	**1642**	**222**	**43**	95, 161, 121, 204, 79	**-**	**2.19**	**3.47**	**-**
53	α-Cadinol	**1654**	**222**	**43**	95, 121, 204, 161, 81	**4.23**	**1.80**	**-**	**-**
54	14-hydroxy-(z)-Caryophyllene	**1667**	**220**	**41**	91, 69, 79, 55, 105	**-**	**-**	**-**	**1.19**
55	14-hydroxy-9-epi-(E)-Caryophyllene	**1669**	**220**	**41**	91, 79, 69, 105, 55	**-**	**-**	**-**	**2.52**
56	epi- α-Bisabolol	**1684**	**222**	**43**	69, 109, 119, 93, 55	**-**	**1.20**	**-**	**-**
57	(Z)- α-trans-bergamotol	**1690**	**220**	**93**	119, 43, 55, 79, 107	**-**	**0.77**	**-**	**-**
58	(2Z,6Z)-Farnesol	**1698**	**222**	**69**	41, 93, 81, 55, 107	**-**	**-**	**-**	**2.01**
59	Mintsulfide	**1741**	**236**	**123**	79, 112, 41, 236, 91	**-**	**-**	**0.22**	**-**
60	(2E,6E)-Farnesol	**1743**	**222**	**69**	41, 81, 93, 55, 107	**-**	**-**	**0.17**	**-**

Table 4: Continue.

No	Name [a]	KI [b]	Mass spectra			Area%			
			MW	B	m/z	*A. c.*	*A. g.*	*A. m.*	*A. s.*
61	Guaiazulene	**1780**	**198**	**183**	198, 153, 168, 128,	**0.15**	-	-	-
62	Isopropyl myristate	**1829**	**270**	**43**	60, 102, 228, 73, 211	**0.11**	-	-	-
63	8,9-Dehydro-cycloisolongifolene	c	**202**	**159**	131, 118, 105, 41, 69	**1.34**	-	-	-
64	Phytol	**1943**	**296**	**71**	43, 55, 82, 123, 95	**0.81**	**1.03**	**0.30**	-
65	n-Tetracosane	**2400**	**338**	**57**	43, 71, 85, 99, 113	-	-	-	**0.15**
66	n-Pentacosane	**2500**	**352**	**57**	43, 71, 85, 99, 113	-	-	**0.1**	**0.93**
67	Heptacosane	**2700**	**380**	**57**	43, 71, 85, 99, 113	-	-	-	**0.49**
68	Nonacosane	**2900**	**408**	**57**	43, 71, 85, 99, 113	-	-	-	**0.25**
	Total identified components (%)					**90.92**	**94.73**	**97.74**	**85.12**
	Oxygenated compounds					**8.39**	**12.05**	**11.92**	**11.43**
	Non-oxygenated compounds					**82.53**	**82.68**	**85.82**	**73.69**
	Monoterpenes					**2.54**	**7.54**	**55.05**	**1.60**
	Sesquiterpenes					**87.57**	**86.16**	**42.29**	**81.7**
	Diterpenes					**0.81**	**1.03**	**0.40**	**0.15**
	Sesterterpenes					-	-	-	**1.67**
	Hydrocarbons					**82.53**	**82.68**	**85.82**	**73.69**
	Alcohols					**8.28**	**7.83**	**9.86**	**2.24**
	Ketones					-	-	**0.79**	-
	Esters					**0.11**	-	**0.53**	**0.17**
	Aldehydes					-	-	**0.74**	-
	Oxides					-	**4.22**	-	**9.02**

A.c.; Annona cherimola, A. g.; Annona glabra, A. m.; Annona muricata, A. s.; Annona squamosa
[a] Compounds are listed in order of their elution time from a DB-5 column.
[b] RI = retention index as determined on DB -5 column based on a homologous series of normal alkanes and by using (Adams, 2009)
[c] Structure confirmed by (Willey7N; Mass Finder and Willey 7 Nist 05; Mass Finder) libraries without determination of their retention indices due to no available standard compound in retention index libraries.

Of the sixty-eight compounds identified in this study, there were only five compounds that were common to all of the *Annona* species except for *A. squamosa* namely; α-copaene, germacrene D, γ-cadinene, δ-cadinene and phytol and they have been suggested to be chemotaxonomic markers for most of Annonaceae (Lima *et al*., 2003)., (Lima *et al*., 2004), (Maia, *et al*.,2005a). In the present work, we found spathulenol in the leaf oils of *A. muricata* only and caryophyllene oxide in *A. glabra* and this in agreement with (Lima *et al*., 2003), (Lima *et al*., 2004), (Maia, *et al*.,2005a) (Maia, *et al*.,2005b).

3.3. Cytotoxic activity

Many researchers are now engrossed in examining the use of herbal medicines as a health care method (Newall *et al.*, 1996). Herbal medicines pursue to be accepted forms of treatment in the Orient, and the plant derived drugs based on traditional practices represent a huge proportion of the pharmaceutical production in modern Western countries (Schulz *et al*., 1998). Development of biologically addressed agents that exploit differences between cancerous and normal cells and grant greater specificity for cancer cells with less damage to normal cells is still the ultimate goal in the field of antineoplastic drug discovery (Adams, 2001).

Ethanol extracts showed highly significant cytotoxic activities (low IC50) much more than results displayed by essential oils on breast (MCF-7), colon (CACO-2) and liver (HEPG2) carcinoma cell lines. Results are presented in table 5.

Regarding breast carcinoma cell line (MCF-7), the IC50 was 3.43, 3.89 and 4.34 µg/ml for *Annona cherimola, A. squamosa* and *A. muricata* ethanol extractives respectively. While colon carcinoma cell line (CACO) displayed IC50 values 2.82, 2.97, 3.58 and 3.89 µg/ml for *A. muricata*, *A. cherimola, A. glabra* and *A. squamosa*, respectively. Liver carcinoma cell line (HEPG2) exhibited IC50 values of 3.12, 3.43 and 3.73 for *A. squamosa*, *A. muricata* and *A. cherimola*, respectively.

Three of the four leaf essential oils showed notable *invitro* cytotoxic activity. Essential oils of *A. glabra, A. muricata and A. squamosa* showed moderate cytotoxic activities with IC50 values ranging from 12.35 to 24.21µg/ml. While the essential oils of *A. cherimola* showed IC50 values ranging from 7.67 to 9.22µg/ml which is highly promising.
To sum up; the essential oil and ethanol extract of *A. cherimola* showed the highest activities on the three carcinoma cell lines tested. These results, compared to Doxorubicin, suggest the highly promising antitumor activity of herbal medicines with especial view to *A. cherimola*.

Doxorubicin is one of the best chemotherapeutic systemic drugs which work well with a variety of other chemotherapy agents, including epirubicin, mitoxantrone, cisplatin and etoposide (Shah *et al*., 1998). It is often used in patients with breast and liver cancer disseminated beyond the liver, although the response rates are generally only 15%. Moreover, doxorubicin is not only expensive but also has serious side effects ranging from nausea, vomiting, ulceration and necrosis of the colon to acute myeloid leukemia with a preleukemic phase and heart failure (Mehta, 2006).

Table 5: IC_{50} of essential oils and ethanol extracts of the four *Annona* species with different cell lines

Tumor cell line	Breast carcinoma cell line (MCF7)		Colon carcinoma cell line (CACO-2)		Liver carcinoma cell line (HEPG2)	
Plant	E.O	Alc. E	E.O	Alc. E	E.O	Alc. E
	IC50 (µg/ml)					
A. cherimola	9.22	3.43	8.43	2.97	7.67	3.73
A. glabra	20.31	18.20	24.21	3.58	14.78	13.3
A. muricata	12.35	4.34	15.34	2.82	17.34	3.43
A. squamosa	13.39	3.89	16.23	3.89	14.27	3.12

Doxorubicin [IC50 (0.42µg/ml for **(MCF-7)**, 1.43µg/ml for **(CACO)** and 1.12µg/ml for **(HEPG2)]**
E.O: essential oil; **Alc. E**: ethanol extract.

Plant bioactive derived compounds are normally cheaper and cause fewer side effects with respect to chemotherapy. Thus, the search for drugs extracted from plant as potential cytotoxic agents for breast, colon as well as liver carcinomas is an important line of research in the discovery of novel anticancer candidates. The plant *A. cherimola* is a well known source of cytotoxic compounds and previously acetogenins have been reported. Annomolin and annocherimolin were isolated from *Annona* species (Kim *et al*., 2001). Annomolin was selectively cytotoxic against the human prostate tumor cell line (PC-3) (Bode and Dong, 2009; Newmann and Cragg, 2007).

3.4. Antimicrobial activity

In the present study, based on previous reports we have found that among the essential oils as well as ethanol extracts four *Annona* species showed wide range of antibacterial and antifungal activities. Generally, volatile oils of all four *Annona* species were more superior to ethanol extracts as antimicrobial. Volatile oils of the four *Annona* species showed an interesting moderate antibacterial activity with *A. glabra* being the most active showing broad spectrum activity against Gram-positive (9-19mm) and Gram-negative bacteria (9-23mm). These results comply with those reported by Rios *et al.* (2003).

It also showed significant antifungal activity against *Candida albicans* with *A. cherimola* being the most active; however, data revealed that All *Annona* species was ineffective and have no antifungal activity against *Aspergillus flavus.*

Table 6: Antimicrobial activity of essential oils and extractives of Annona species

Microorganism	*Gram reaction*	Inhibition zone diameter (mm)									
		Standard		*Annona cherimola*		*Annona glabra*		*Annona muricata*		*Annona squamosa*	
		Gentamycin	AmphotericinB	E.O.	Alc.E	E.O.	Alc.E	E.O.	Alc.E	E.O.	Alc.E
***Bacillus subtilis* (ATCC 6051)**	G^+	33	--	15	10	19	12	18	17	16	9
***Staphylococcus aureus* (ATCC 12600)**	G^+	30	--	14	9	17	11	15	11	17	13
***Escherichia coli* (ATCC 11775)**	G^-	33	--	14	9	22	12	19	13	15	14
***Pseudomonas aeruginosa* (ATCC 10145)**	G^-	32	--	16	10	23	14	20	12	16	11
***Aspergillus flavus* Link (ATCC 204304)**	Fungus	--	16	≤6	≤6	≤6	≤6	≤6	≤6	≤6	≤6
***Candida albicans* (ATCC 26555)**	Fungus	--	18	13	7	14	9	16	14	12	8

Results are average of three replicate tests; E.O., Essential oil; Alc.E, ethanol extract.

The **MIC** of the four *Annona* leaves essential oils (Table 7) revealed variability in the inhibitory concentrations of each essential oil for the given microorganisms.

The essential oils of leaves showed activities in the range (concentrations) from 110 to 285 µg/ml for *A. cherimola*, 30 to 97µg/ml for *A. glabra*, 31 to 112µg/ml for *A. muricata* and from 52 to 304 µg/ml for *A. squamosa*. Alcoholic extractives showed higher MIC values than those of respective essential oils.

Table7: Minimal Inhibitory Concentration (MIC) of essential oils and extractives of Annona species against bacterial and fungal strains.

Tested microorganisms	MIC (µg/ml)							
	A. c.		*A. g.*		*A. m.*		*A. s.*	
	E.O.	Alc.Ex	E.O.	Alc.Ex	E.O.	Alc.E	E.O.	Alc.E
***Bacillus subtilis* (ATCC 6051)**	130	220	65	140	69	162	110	180
***Staphylococcus aureus* (ATCC 12600)**	285	315	37	110	112	172	304	110
***Escherichia coli* (ATCC 11775)**	110	150	30	112	31	126	52	182
***Pseudomonas aeruginosa* (ATCC 10145)**	140	125	97	111	104	171	109	101
***Aspergillus flavus* Link (ATCC 204304)**	ND	ND	ND	ND	ND	ND	ND	ND
***Candida albicans* (ATCC 26555)**	152	176	64	121	100	142	133	151

A. c.; Annona cherimola, A. g.; Annona glabra, A. m.; Annona muricata, A. s.; Annona squamosa,
E.O.; essential oil, Alc.E; Alcohol extract,
ND; Not done, as essential oil (s) or extract has no antimicrobial activity on this microorganism.

The lowest variation (30µg/ml and 31µg/ml) was observed for essential oils of *A. glabra* and *A. muricata*, respectively, on *Escherichia coli* that may be attributed to their higher oxygenated content of their essential oils. These results are in agreement with Pandey and Barve (2011) who documented a MIC varying from 130 to180 µg/ml when testing different concentrations of *Annona* extractives on both Gram-negative and Gram-positive bacteria.

4. Significance and impact of the study

This is the first report on cytotoxic and antimicrobial characterization of essential oils as well as ethanol extracts of Egyptian *Annona* species and their relation with its constituents and DNA fingerprint with great potential for future applications.

5. Disclosure policy

The authors declare that no conflict of interests regarding the publication of this article.

6. Conclusion

The extensive survey literature reviewed that *Annona* species, are important medicinal plants with diverse pharmacological spectra. Few novel chemical constituent isolated from the *Annona* species showed anticancer, anti-HIV and antidiabetic (type 2 diabetic) properties too (Pandey & Barve 2011). DNA phylogenetic analysis has suggested that the diversification of the genus *Annona* has no direct relation to cytotoxic activity and antimicrobial activity of essential oils or their extractives. Further evaluation need to be carried out on *Annona* species in order to explore concealed areas and their practical clinical application, which can be used for the welfare of the mankind.

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