Cytotoxic essential oil from *Annona sengalensis* Pers. leaves

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ABSTRACT

The cytotoxicity against brine shrimp of the essential oil obtained from the leaves of *Annona senegalensis* Pers. (Annonaceae) was studied. The confirmation of this toxicity has been done by using selected tumor cell lines (A549, HT29, MCF 7, RPMI, and U251). The results showed that the total oil and its fractions have showed mild to moderate cytotoxicity in brine shrimp lethality bioassay with LC50 = 27.3 μg/ml, and against some human tumor cell lines. The total oil and its fractions were analyzed by gas chromatography/mass spectroscopy (GC/MS). Seventy three compounds were identified.

Key Words: *Annona senegalensis*, GC/MS, essential oil, brine shrimp, cytotoxicity

INTRODUCTION

Annona senegalensis is found widely distributed in Africa, Latin America, and Europe. The plant possesses several folk medicinal uses. The root bark is used for intestinal troubles and the bark is chewed for stomach ache. The stem, root, and bark are used to treat diarrhoea and gastrointestinal trouble,[8] whereas the stem bark and leaves are used for the treatment of skin cancer and leukemia.[9,10]

In this study, the essential oil of *A. senegalensis* var. *Senegalensis* was studied for its cytotoxicity as well as its chemical composition, where 19 mono and sesquiterpenoids were identified in the volatile oil of the leaves and fruits.[11] No reports have been found in the literature on the biological activity of *A. senegalensis* essential oil. On these bases, the volatile oil of *A. senegalensis,* cultivated in Egypt, was prepared analyzed by gas chromatography/mass spectroscopy (GC/MS) and was screened for possible anticancer activity.

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MATERIALS AND METHODS

Plant material

The leaves of *A. senegalensis* Pers. were collected from El-Qanater garden, Qalubeia Province, Egypt, and identified by Agric. Eng. Badia Diwan, Herbarium of Orman Botanical Garden, Giza.

Essential oil preparation

Exactly 2 kg of fresh leaves were sliced in small pieces (2–3 cm^2), and hydro-distillation was performed according to Egyptian Pharmacopoeia 1984. The essential oil was obtained in 0.021% w/w.

Analysis of volatile oil

Thin layer chromatography (TLC): The analysis was carried out on silica gel 60 F_{254}, precoated plates, layer thickness: 250 μm (E. Merck, Darmstadt, Germany) and developed in different systems where Benzene:EtOAc (86:14) gave the highest resolution. The spots were visualized by UV and *P*-anisaldehyde/H_2SO_4 reagent.

Flash column chromatography: 400 mg of the volatile oil was chromatographed on a column (1.5 × 25 cm) using silica gel (5–40 μm) and benzene:EtOAc (86:14) as eluting solvent, where 50 fractions (5–10 ml each) were collected and combined into three pools as guided by TLC. The pools after evaporation yielded P-A (105 mg), P-B (45 mg), and P-C (40 mg). The fractionation of P-A was effected over

silica gel column (5–40 µm), using benzene:EtOAc (86:14); 50 fractions (3–5 ml each) were collected and combined into six pools (PA1–PA6) as guided by TLC.

GC/MS: GC/MS analysis were carried out using a Hewlett Packard 5890A-5970 GC-MS series with mass selective detector, 9144 HP 5Ms (crosslinked 5% PHME siloxane) 30 m × 0.25 µm, film thickness HP-5, 50°C for 2 min, then 0°C/min to 270°C, injecting temperature: 270°C; carrier gas He 20 ml/min, injecting volume: 25 µl, MS: in the EI mode at 70 eV, det.: 2300 IMEM, scan range: 47–400.

Cytotoxicity bioassays

Brine shrimp: A solution of sea water was made by dissolving 32.5 g (a natural blend of salts and trace element for sea water fish [Sera Company, Aquaristik Gmbh, D5138 Henisberg, Germany]) in distilled water (1 l). *ca.* 1 mg of brine shrimp, *Artemia salina* (leach), eggs was taken in a hatching chamber (22 × 32 cm). The hatching chamber was kept under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae (nauplii). Then 50 mg of tested extracts/fractions; or 1 mg of pure compounds, dissolved in 5 ml of solvent in which they were soluble and from this, 5, 50, and 500 µl of each solution was transferred to vials corresponding to 10, 100, and 1000 µg/ml, respectively. Each dosage was tested in triplicate. The test vials and one control containing 500 µl of solvent were allowed to evaporate to dryness under nitrogen. Ten larvae (nauplii) of *A. salina* were transferred into each vial and the volume made into 5 ml with sea salt solution (Dimethyl sulfoxide, DMSO) immediately after adding the nauplii, 24 h later, the number of surviving shrimp at each dosage was counted and recorded. LC_{50} values were determined statistically.[12]

Human tumor cell cytotoxicity assay (HTCC)

This cytotoxicity assay was carried out at the Ohio State University Comprehensive Cancer Centre with the cooperation of Professor John Cassady. From growing stock cultures, cells were inoculated into 96-well tissue culture plates on day one (D1) at appropriate concentrations (1000–2000 cells depending on the cell line), then incubated for 24 h. Test compounds were then added on day two (D2) in five log dilutions beginning with the highest soluble concentration, (four wells for each concentration). Simultaneously, negative controls (no treatment) and positive controls (adriamycin, five log dilutions) are included then ED_{50} (Dose of a drug that is pharmacologically effective for 50% of the population exposed to the drug) was as calculated.[4]

RESULTS

The steam volatile dark yellow oil isolated from the fresh leaves of *A. senegalensis* Pers., cultivated in El-Qanater garden, Egypt, showed mild to moderate cytotoxicity in brine shrimp lethality bioassay with LC_{50} = 27.3 µg/ml, and against some human tumor cell lines (HTCL) (*cf.* Table 1). Preliminary TLC analysis of the oil performed on pre-coated silica gel 60 F_{254}, showed an imaginable condensed pattern, as detected with the UV or spraying

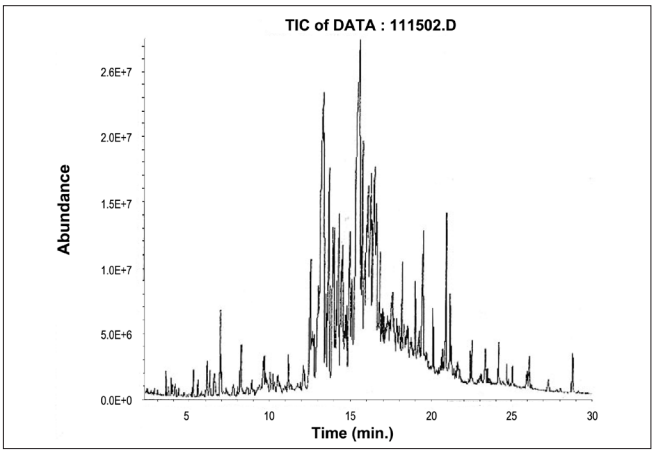

Figure 1: Gas chromatogram of the total volatile oil of *A. senegalensis* leaves

Table 1: Cytotoxic activity of the total volatile oil and its fractions on HTCL

VO pools	ED50 (µg/ml)				
	A549	HT29	MCF 7	RPMI	U251
Total volatile oil	3 × 10−1	1 × 10°	1 × 10−1	2 × 10−2	1 × 10−1
Pool-A	NT	NT	NT	NT	NT
Pool-B	1 × 10°	1 × 10°	8 × 10−2	5 × 10°	7 × 10−2
Pool-C	4 × 10−2	1 × 10°	5 × 10−2	1 × 10°	2 × 10−2
Fractions of P-A					
PA1	1 × 10°	2 × 10°	2 × 10−2	6 × 10−1	3 × 10−1
PA2	2 × 10−1	1 × 10°	1 × 10−1	2 × 10−1	1 × 10−1
PA3	9 × 10−1	1 × 10−1	1 × 10−1	7 × 10−1	1 × 10−1
PA4	6 × 10−1	6 × 10−1	3 × 10−2	3 × 10−1	7 × 10−2
PA5	IA	IA	IA	IA	IA
PA6	9 × 10−1	9 × 10−1	5 × 10−1	7 × 10-1	3 × 10−1

A549, human lung carcinoma; HT29, human colon adenocarcinoma; MCF7, human breast adenocarcinoma; RPMI, malignant melanoma; U251, glioblastoma multiform; NT, not tested; IA, inactive (ED_{50} >100 µg/mL).

Table 2: Compounds identified in the total volatile oil and fractions of *A. sengalensis* Pers. by GC/MS analysis

Compound	M. Wt.	CF	Detected in
Furancarboxyldehyde	96	$C_5H_4O_2$	VO
1-Limonene	136	$C_{10}H_{16}$	VO
Camphene	136	$C_{10}H_{16}$	VO
2(5H) Furanone, 3-methyl	98	$C_5H_6O_2$	VO
β-myrcene	136	$C_{10}H_{16}$	VO
Para-cymene	134	$C_{10}H_{14}$	VO
Terpineol	154	$C_{10}H_{18}O$	VO
Para-cymene-8-ol	150	$C_{10}H_{18}O$	VO
Endo bornyl acetate	196	$C_{12}H_{20}O_2$	VO
Alpha-copaene	204	$C_{15}H_{24}$	VO, PA1, PA2
Beta-elemene	204	$C_{15}H_{24}$	VO
Caryophyllene (*trans*)	204	$C_{15}H_{24}$	VO, PA2, PA5,
Z,E-alpha-farnesene	204	$C_{15}H_{24}$	VO
Alpha-humulene	204	$C_{15}H_{24}$	VO, PA1
7,11-Dimethyl,9-methylene,-1,6,10-dodecatriene	190	$C_{15}H_{10}$	VO
Beta-bisabolene	204	$C_{15}H_{24}$	VO, PA1, PA2, PA5
Carotol	222	$C_{15}H_{26}O$	VO, PA4
Caryophyllene oxide	220	$C_{15}H_{26}O$	VO, PA2, PA3
Virdiflorol	222	$C_{15}H_{26}O$	VO, P-B
Nerolidol (*cis*)	220	$C_{15}H_{26}O$	VO, PA5
β-Santalol	220	$C_{15}H_{24}O$	VO
2-Penta decanone 6,10,14-triethyl	268	$C_{18}H_{36}$	VO
1,2-Benzene dicarboxylic acid butyl ester	278	$C_{16}H_{22}O_4$	VO, PA3, PA5
2-Hexadecen-1-ol,3,7,11,15-tetramethyl	296	$C_{20}H_{42}O_4$	VO
Hexatriacontane	206	$C_{30}H_{24}$	VO
Eicosane	282	$C_{20}H_{42}$	VO
Pentatriacontane	492.	$C_{35}H_{72}$	VO
Alpha-cubebene	204	$C_{15}H_{24}$	PA1
Iso-caryophyllene	204	$C_{15}H_{24}$	PA1
Beta-gurjunene	204	$C_{15}H_{24}$	PA1
trans-beta–Farnesene	204	$C_{15}H_{24}$	PA1
Gama-cadinene	204	$C_{15}H_{24}$	PA1, PA2
Delta-cadinene	204	$C_{15}H_{24}$	PA1
Alpha-muurolene	204	$C_{15}H_{24}$	PA1
Eicosane,7-hexyl	366	$C_{26}H_{54}$	PA1
Tricosane	324	$C_{23}H_{48}$	PA1
Pentacosane	352	$C_{25}H_{52}$	PA1, PA5
Hexacosane	366	$C_{26}H_{54}$	PA1
Heptacosane	380	$C_{27}H_{56}$	PA1
2,4-Di-isopropenyl-1-vinyl-cyclohexane	204	$C_{15}H_{24}$	PA2
Bergamotene [Z]-alpha-*trans*	204	$C_{15}H_{24}$	PA2
Beta-farnesene	204	$C_{15}H_{24}$	PA2
Alpha-epi-bisabolol	222	$C_{15}H_{26}O$	PA2, PA4, PA6
Torreyol	222	$C_{15}H_{26}O$	PA2
Cyclohexanol, 4-chloro-*trans*	136	$C_6H_{11}ClO$	PA3, PA4, PA5, PA6
1,2-Benzene dicarboxylic bis (2-ethyl hexylester)	390	$C_{24}H_{38}0$	P-C
2-Cyclo hexane-1-one	96	C_6H_80	PA5, PA6
Ethanol, 2-ethoxy-acetate	132	$C_6H_{12}O_3$	PA5
Oxirane, 2-methyl-3-propyl	100	$C_6H_{12}O$	PA5
Benzamine, 3,5-dichloro	164	$C_6H_5Cl_2N$	PA5
Gossonorol	218	$C_{15}H_{22}O$	PA5
9-Octadecenamide (*Z*)	281	$C_{18}H_{35}NO$	PA5
1,2-Benzene dicarbaxylic, 3-nitro	211	$C_8H_5NO_6$	PA5

Table 2: Contd...

Compound	M. Wt.	CF	Detected in
Propanol, 2-hydroxy-2-methyl	80	$C_4H_8O_2$	PA6
2-Pentanol, 4-methyl	98	$C_6H_{14}O$	PA6
2H-pyran-2-one	98	$C_5H_4O_2$	PA6
Benzamine 3,4 dichloro	164	$C_6H_5Cl_2N$	PA6
Phenol 2,6-bis (1,1-dimethyl methyl)-4-methoxy	220	$C_{15}H_{24}O$	PA6
Trans-nerolidol	220	$C_{15}H_{26}O$	PA6
Alpha-cadinol	222	$C_{15}H_{26}O$	PA6
Phthalic acid butyl ester (ester with butylglucolate	336	$C_{18}H_{24}O_6$	PA6
4(2,2′-dimethyl 2′-methyl dencyl ethoxy butanol	194	$C_{13}H_{22}O$	PA6
2-Hexadecen-1-al,7,9,11,15-tetramethyl)	294	$C_{20}H_{38}O$	PA6
2- (2′-hydroxyethl) cyclopentanone	128	$C_7H_{12}O_2$	PA6
Di-(2-ethylhexyl) ester of adipic acid	370	$C_{22}H_{24}O_4$	PA6
Bergomotol <[Z]-alpha>	220	$C_{15}H_{24}O$	P-B
Ether, 1-hexadecenyl methyl	254	$C_{17}H_{34}O$	P-B
1,2-Benzene dicarboxylic butyl phenyl methyl ester	312	$C_{19}H_{20}O_4$	P-C
Dodecane	170	$C_{12}H_{26}$	P-C
Nonacosane	408	$C_{29}H_{28}O_2$	P-C
Spathulenol	220	$C_{15}H_{25}O$	P-C
Hexodecanoic acid	256	$C_{16}H_{34}O_2$	P-C
Acetoxylemol <8-alpha>	320	$C_20H_{22}O_3$	P-C

VO, volatile oil (total); P-B and P-C, VO pooled fractions; PA1–PA6, pooled fractions from PA.

with anisaledehyde sulfuric acid reagent. GC analysis of the volatile oil afforded 181 peaks [Figure 1], of which only 27 compounds were identifiable by GC/MS analysis, as aided by data library and confirmed by comparison with published MS spectra.[8] To concentrate the minor components, the oil was fractionated on NP silica gel, eluted with benzene:EtOAc (6:4), and the obtained fractions grouped into three pools (P-A least polar, P-B, and P-C most polar) as guided by TLC. At 50 ppm, P-A showed the highest cytotoxicity (93%), in the brine shrimp lethality bioassay, whereas P-B and P-C showed 21 and 40% cytotoxicity, respectively. Thus, P-A was selected for further fractionation, which resulted in six pools possessing variable lethality percentages on brine shrimp larvae at 50 ppm as follows: PA1, PA2, and PA5 gave 0%; PA3: 12.5%, PA4: 49%, and PA6: 45%, and on HTCL *in vitro* as clarified in Table 1. The six pools were further reanalyzed by GC/MS. The interpretation of the GC/MS chromatograms led to identification of 46 other compounds. The total 73 components identified in the volatile oil and d its fractions are grouped in Table 2.

DISCUSSION

The main component identified in the moderately cytotoxic

fraction P-4 is caryophyllene oxide (64.5%), which when tested in pure form proved to be devoid of cytotoxicity on the five HTCL tested, with moderate cytotoxicity on brine shrimp (LD_{50} 36.8 ppm). As the bioactivity did not increase significantly in the fractions, it could be safely concluded that the mild to moderate cytotoxicity of this volatile oil is apparently due to a synergistic effect exerted by its particular combination of oxygenated and nonoxygenated monoterpenes, and sesquiterpenes as well as the other components.

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