

SUPPLEMENTARY MATERIAL

Chemical composition of the essential oil from the leaves of *Anaxagorea brevipes* (Annonaceae) and evaluation of its bioactivity

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The essential oil obtained by hydrodistillation from leaves of *Anaxagorea brevipes* was analyzed by Gas Chromatography fitted with a flame ionization detector (GC-FID) and coupled to mass spectrometry (GC-MS). Thirty one components were identified, representing around 75.7% of total oil. The major components were β -eudesmol (13.16%), α -eudesmol (13.05%), γ -eudesmol (7.54%), guaiol (5.12%), caryophyllene oxide (4.18%) and β -bisabolene (4.10%). The essential oil showed antimicrobial activity against Gram-positive bacteria and yeast with the MIC values between 25.0 and 100 μ g/mL. The highest antiproliferative activity was observed for the oil against MCF-7 (breast, TGI = 12.8 μ g/mL), NCI-H460 (lung, TGI = 13.0 μ g/mL) and PC-3 (prostate, TGI = 9.6 μ g/mL) cell lines,

while against no cancer cell line Hacat (keratinocyte) the TGI was 38.8 $\mu\text{g/mL}$. The oil exhibited a small antioxidant activity assessed through ORAC-FL assay (517 $\mu\text{mol TE/g}$). This is the first report regarding chemical composition and bioactivity of *A. brevipes* essential oil.

Keywords: *Anaxagorea brevipes*; Annonaceae; essential oil; bioactivity.

1. Experimental

1.1. Plant material

Leaves of *A. brevipes* were collected in September 2009, the morning (at 9:00 hours), in the “Campus of the Federal University of Amazonas”, Municipality of Manaus, Amazonas State, Brazil, coordinates: [S 03° 05’ 05.5” and W 059° 57’ 29.3”]. The species were identified by Dr. Antonio Carlos Webber, a plant taxonomist of the Department of Biology of the institute of Biological Sciences of the Federal University of Amazonas (DB/ICB/UFAM). A voucher specimen number 8280 was deposited at the Herbarium of the UFAM. Leaves were obtained from flowering species.

1.2. Hydrodistillation of the volatile constituents

The essential oil was obtained by hydrodistillation. Fresh leaves (pool of the samples) taken from different parts of *A. brevipes* (300 g) were dried with circulating air at 45°C for 24 hours and submitted to hydrodistillation in a Clevenger-type apparatus for 3 hours. The essential oil was recovered with dichloromethane and dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the percentage content (v/w) was calculated in relation to the dry weight of the plant material. The oil was stored in a freezer until analysis. The hydrodistillation was performed in duplicate.

1.3. Gas Chromatography analysis

GC analysis: GC analyses were carried out using a Shimadzu GC-17A fitted with a flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a ZB-5MS fused capillary column (30 m X 0.25 mm X 0.25 μm film thickness) coated with 5%-phenyl-arylene-95%-methylpolysiloxane. Helium was the carrier gas at 1.2 $\text{mL}\cdot\text{min}^{-1}$ flow rate. The column temperature program was: 50°C/2 min, a rate of 4°C/min to 200°C, then a rate of 15°C/min to 300°C, and then 300°C/15 min. The injector and

detector temperatures were 250°C and 280°C, respectively. Samples (0.5 µL in CH₂Cl₂) were injected with a 1:100 (v/v) split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C₈-C₁₈). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction.

GC/MS analysis: GC/MS analyses were performed on a Shimadzu QP5050A GC/MS system equipped with an AOC-20i auto-injector. A J&W Scientific DB-5MS (coated with 5%-diphenyl-95%-dimethylpolysiloxane) fused capillary column (30 m X 0.25 mm X 0.25 µm film thickness) was used as the stationary phase. MS were taken at 70 eV with scan interval of 0.5 s and fragments from 40-500 Da. The other conditions were similar to the GC analysis.

Identification of constituents: Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds ran under identical conditions, and by comparison of retention indices (van Den Dool H and Kratz, 1963) and MS (Adams, 2007) with those in the literature, and by comparison of MS with those stored in the NIST and Wiley libraries.

1.4. Evaluation of antibacterial and antifungal activities in vitro

Essential oil of *A. brevipes* was evaluated for antimicrobial activity using the broth microdilution method (96-well microtiter plates), as previously described by Salvador et al. (2002), to give concentrations between 12.5 to 500 µg/mL. The minimal inhibitory concentration (MIC) was calculated as the lowest concentration showing complete inhibition of the tested strain. In these tests, bacitracin and ketoconazole were used as experimental positive controls for bacteria and fungi strains respectively, while the solution of propyleneglycol/sterile distilled water (5:95, v/v) served as the negative control. Each sensitivity test was performed in duplicate for each microorganism evaluated and the results were presented as arithmetic averages. The strains of microorganisms utilized are shown in Table S2.

1.5. Antiproliferative assay

Cell lines: U251 (glioma, CNS), UACC- 62 (melanoma), MCF-7 (mama), NCI-ADR/RES (ovarian-resistant), NCI-H460 (lung, no small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), K562 (leukemia) and VERO cell lines, from American Type Culture

Collection (ATCC), were used. The assay was done as described previously (Skehan et al., 1990). Briefly, the cells were distributed in 96-well plates (100 μ L cells/well) and exposed to various concentrations of essential oil (0.25, 2.5, 25.0 and 250.0 μ g/mL) in DMSO (0.1%) at 37 °C, with 5% of CO₂, for 48 h. The final concentration of DMSO did not affect the cell viability (negative control). Trichloroacetic acid (50% solution) was added and after incubation for 30 min at 4°C, plates were washed with water and left to dry at room temperature. Cell proliferation was determined by spectrophotometric quantification (at 540 nm) of the cellular protein content using sulforhodamine B. The experiments were carried, at least, in triplicate and the concentration necessary to total growth inhibition (TGI) was calculated in μ g/mL. Doxorubicin was used as positive control. Using the concentration-response curve for each cell line, TGI (concentration that produces total growth inhibition or cytostatic effect) was determined through non-linear regression (sigmoidal fitting) analysis using the software ORIGIN 8.0 (OriginLab Corporation, Northampton, MA, USA) [28].

1.6. TLC autographic assay for DPPH Radical-Scavenging

In this assay, 1:250, 1:100 and 1:50 (v/v), dilution of the essential oil in dichloromethane were made and then 10 μ L of each dilution were applied on TLC plates (silica gel 60 GF254, Fluka, AG, Switzerland). The plates were sprayed with a 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St Louis, MO) solution, in MeOH, and left at room temperature. Plates were observed 30 min after spraying. Active samples were observed as yellow spots against a purple background. Relative radical-scavenging activity was assigned as “strong” (samples that produced an intense bright yellow zone), “medium” (samples that produced a clear yellow spot), “weak” (samples that produce only a weakly visible yellow spot), or “not active” (samples that produced no sign of any yellow spot) (Cavin et al., 1998).

1.7. ORAC_{FL} kinetic assay

The antioxidant capacity of the essential oil was assessed through the oxygen radical absorbance capacity (ORAC_{FL}) assay. This assay is based upon the inhibition of the peroxy radical-induced oxidation initiated by the decomposition of the peroxy radical (2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH, Aldrich, Milwaukee, WI), using fluorescein (Aldrich, Milwaukee, WI) as the fluorescent probe. The assay was carried out on a Synergy 2 (Biotek, Winooski, VT) multi detection microplate reader system. The temperature of the

incubator was set at 37°C and the procedure performed according to the method described by Huang et al. (2002). The data are expressed (Costa et al., 2011) as µmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich, Milwaukee, WI) equivalents (TE) per gram of oil on a dry basis (µmol of TE/g). The analyses were performed in triplicate.

References

- Adams, R. P. 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed.; Allured Publishing Corporation: Illinois, USA.
- Cavin, O. P., Wolfender, J. L., Hostettmann, K., Dyatmyko, W. 1998. Use of on-flow LC/1H NMR for the study of an antioxidant fraction from *Orophea enneandra* and isolation of polyacetylen. Journal of Natural Products, 61:1497-1501.
- Costa, E. V., Dutra, L. M., Jesus, H. C. R., Nogueira, P. C. L., Moraes, V. R. S., Salvador, M. J., Cavalcanti, S. C. H., Santos, R. L. C.,; Prata, A. P. N. (2011). Chemical composition and antioxidant, antimicrobial, and larvicidal activities of the essential oils of *Annona salzmannii* and *A. pickelii* (Annonaceae). Natural Product Communications. 6: 907-912.
- Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E. 2002. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. Journal of Agricultural and Food Chemistry, 50:1815-1821.
- Salvador, M. J., Ferreira, E. O., Pral, E. M. F., Alfieri, S. C., Albuquerque, S., Ito, I. Y., Dias, D. A. 2002. Bioactivity of crude extracts and some constituents of *Blutaparon portulacoides* (Amaranthaceae). Phytomedicine, 9:566-571.
- Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H. Kenney, S., Boyd, M. R. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute, 82:1107-1118.
- Van Den Dool, H. , Kratz, P.D. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. Journal of Chromatography, 11:463-471.

Captions

Table S1. Chemical composition (%) of the essential oil from the leaves of *Anaxagorea brevipes*.

Table S2. Antimicrobial activity of the essential oil from the leaves of *Anaxagorea brevipes*.

Table S3. Antiproliferative activity of the essential oil from the leaves of *Anaxagorea brevipes*.

Table S1. Chemical composition (%) of the essential oil from the leaves of *Anaxagorea brevipes*.

Number	Compounds	RI ^a	RI ^b	GC-FID (%)
1	α -Pinene	932	932	1.42
2	Limonene	1029	1024	1.93
3	Isoledene	1380	1374	0.69
5	Aromadendrene	1444	1439	0.45
6	Germacrene D	1482	1484	1.85
7	α -Muurolene	1506	1500	1.96
8	4- <i>epi-cis</i> - Dihydroagarofuran	1508	1499	1.18
9	β -Bisabolene	1510	1505	4.10
10	γ -Cadinene	1517	1513	1.82
11	Kessane	1535	1529	0.45
12	Liguloxide	1537	1536	0.53
13	α -Calacorene	1546	1544	0.40
14	β -Calacorene	1550	1465	1.93
15	<i>epi</i> - Longipinanol	1566	1562	1.60
16	Palustrol	1576	1567	0.82
17	Caryophyllenyl alcohol	1579	1570	1.23
18	Caryophyllene oxide	1591	1582	4.18
19	Viridiflorol	1600	1592	0.85
20	Guaiol	1605	1600	5.12
21	Rosifoliol	1610	1600	1.37
22	5- <i>epi</i> -7- <i>epi</i> - α -Eudesmol	1614	1607	1.19
23	1,10-di- <i>epi</i> -Cubenol	1621	1618	2.05
24	γ -Eudesmol	1629	1630	7.54
25	<i>cis</i> -Cadin-4-en-7-ol	1637	1635	0.52
26	Agarospirol	1643	1646	1.51
27	α -Muurolol	1649	1644	1.71
28	β -Eudesmol	1661	1649	13.16
29	α -Eudesmol	1664	1652	13.05
30	Bulnesol	1676	1670	0.72
31	Cadalene	1684	1675	0.36
Monoterpenes identified				3.35
Sesquiterpene hydrocarbons identified				13.56
Oxygenated sesquiterpenes identified				58.78
Total identified				75.69

RI^a (calc.), retention indices on DB-5MS column calculated according to van Den Dool H, Kratz PD (1963). RI^b retention indices according to Adams (2007).

Table S2. Antimicrobial activity of the essential oil from the leaves of *Anaxagorea brevipes*.

Microorganism	^a MIC ($\mu\text{g mL}^{-1}$)	
	Essential of <i>A. brevipes</i>	Positive controls ^b
<i>Kocuria rhizophila</i> (ATCC 9341) ^c	50.00	50.00 ⁰¹
<i>Staphylococcus aureus</i> (ATCC 14458) ^c	250.00	25.00 ⁰¹
<i>S. aureus</i> penicillinase-negative (8-) ^d	25.00	25.00 ⁰¹
<i>S. aureus</i> penicillinase-positive (7+) ^d	250.00	25.00 ⁰¹
<i>Staphylococcus epidermidis</i> (ATCC 12228) ^c	^e -	50.00 ⁰¹
<i>S. epidermidis</i> (6ep) ^d	-	50.00 ⁰¹
<i>Enterococcus faecalis</i> (Ef) ^d	250.00	50.00 ⁰¹
<i>Escherichia coli</i> (ATCC 10538) ^c	-	50.00 ⁰¹
<i>Pseudomonas aeruginosa</i> (ATCC 27853) ^d	-	>500.00 ⁰¹
<i>Candida albicans</i> (ATCC 10231) ^c	50.00	12.50 ^{b2}
<i>Candida albicans</i> (ATCC 1023) ^c	50.00	12.50 ^{b2}
<i>Candida parapsilosis</i> (ATCC 22019) ^c	100.00	12.50 ^{b2}
<i>Candida dubliniensis</i> (ATCC 777) ^c	>500.00	12.50 ^{b2}
<i>Candida dubliniensis</i> (ATCC 778157) ^c	>500.00	12.50 ^{b2}

^aMIC: minimum inhibitory concentration in $\mu\text{g mL}^{-1}$; ^bPositive controls: ^{b1}chloramphenicol for bacteria strains and ^{b2}ketoconazole for yeast strains; ^cstandard strain; ^dfield strain; ^e-: without inhibition of the development; Compounds evaluated in the range of 12.5 and 500 $\mu\text{g/mL}$ in microdilution method according to Costa et al. (2010).

Table S3. Antiproliferative activity of the essential oil from the leaves of *Anaxagorea brevipes*.

Cell lines	^a TGI ($\mu\text{g mL}^{-1}$)	
	Essential oil of <i>A. brevipes</i>	Doxorubicin (Positive control)
U251	35.15	1.40
UACC-62	94.67	0.28
MCF-7	12.83	0.95
NCI-H460	13.02	0.08
PC-3	9.61	1.30
K562	78.66	4.92
HaCat	38.80	0.37

^aTGI: concentration that causes total growth inhibition in $\mu\text{g mL}^{-1}$. The coefficients of variation obtained in these analyses were below to 5%. Cell lines: U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, no small cells), PC-3 (prostate), K562 (leukemia) and HaCaT (human keratinocyte).