


Headspace solid-phase microextraction/GC/MS of volatile constituents of *Araucaria cunninghamii* and its antimicrobial potentials against multidrug-resistant pathogens

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ABSTRACT

Investigation of the volatile constituents of *Araucaria cunninghamii* Aiton ex D. Don aerial parts was carried out using the headspace/solid-phase microextraction technique using gas chromatography coupled to mass detector. Screening of the phytochemical constituents of the aerial parts *A. cunninghamii* methanolic extract was prepared to standardize their phenolic and flavonoidal content, as well as antimicrobial screening for the extract. Investigation of the volatile constituents of *A. cunninghamii* identified a total of 31 volatiles as monoterpene and sesquiterpene. Standardization of the plant methanolic extract showed its elevated content of total phenolics and total flavonoids. Evaluation of the extract's antimicrobial activities against multidrug-resistant pathogens using the agar well diffusion method was determined at extract concentrations of 100 and 200 mg/ml. The results revealed that the extracts inhibit the growth of the tested Gram +ve spp. with a moderate extent followed by the Gram -ve spp. (-)- α -Pinene was the major monoterpene hydrocarbon representing a third of the volatile constituents of the sample 32.95% and this is concurrent with the plant being one of the conifers' standardization of *A. cunninghamii* which showed a high total polyphenolic content with 252.11 ± 0.28 mg/gallic acid equivalent and exhibited also a high flavonoidal content of 21.65 ± 0.01 mg/ μ g compared to the standard quercetin.

INTRODUCTION

Plants are an integral part of human life and have a major role in biodiversity due to renewed interest in herbal and homeopathic medicines. Plant-derived pharmaceuticals have become prominent in the marketplace, as a favored healthcare choice. The increased popularity of plant-based medicines can also be attributed to the general perception that products from

nature are inherently "safer" due to the history of human use and the long association of plants and humans. Today, there is a wealth of products on the market which contain active plant material (Raskin *et al.*, 2002).

Araucariaceae is a family of large evergreen trees, dioecious or monoecious, with relatively large pith in the trunk and resin in the cortex (Byng, 2015; Welch, 2012). The family contains 3 genera with 39 species (Mabberley *et al.*, 1997) including *Araucaria* as one of its genera. This species has excellent timber, which is used for wood production and used for furniture work and flooring (Bein *et al.*, 1996) and the resin yielded from the tree trunk is used in ulcer treatment and wound healing; in addition, its seeds are eaten, and it is a human source of edible nuts providing a source of valuable proteins such as *Araucaria cunninghamii*

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seeds which have a taste like cashew. *Araucaria* is an endangered species due to its exposure to extensive logging by humans for its importance as industrial crops.

Araucaria species are known to possess gastroprotective (Schmeda-Hirschmann *et al.*, 2005), antidepressant (Vasconcelos *et al.*, 2009), antiviral (Freitas *et al.*, 2009), anti-inflammatory (Santi-Gadelha *et al.*, 2006), neuroprotective (Mukherjee *et al.*, 2007), antipyretic (Suresh *et al.*, 1995), anticoagulant (Dhanasekaran *et al.*, 1993), and antimicrobial (Cespedes *et al.*, 2006) activities. The genus is native to Papua New Guinea, Australia, New Caledonia, and South America. *Araucaria cunninghamii* Aiton ex D. Don are grown as ornamental trees in streets, parks, and gardens and was the target of the current study.

MATERIALS AND METHODS

Plant material for solid-phase microextraction (SPME) volatiles isolation

The leaves of *A. cunninghamii* Aiton ex D. Don were collected from Prince Mohamed Ali Palace Garden in AL-Manyal, Giza, Egypt. The identity of the plant was authenticated by Prof. Dr. Abd El-Haleem Abd El-Motgali at Flora and Phytotaxonomy Department, Horticultural Researches Institute, Agricultural Research Center, Dokki, Cairo, Egypt. Headspace volatiles analysis using SPME was adopted from a previous study (Zini *et al.*, 2002) with few modifications. *Araucaria* aerial parts were ground, and 100 mg was placed in a 1.5 ml glass vial. The 0.1 M sodium phosphate buffer solution (pH 7.2 ml) was added to the vial, which was then immediately capped and placed on a temperature-controlled tray for 30 minutes at 40°C with the SPME fiber inserted into the headspace above the plant sample. Adsorption was timed for 30 minutes. SPME fibers were desorbed at 210°C for 1 minute in the port of injection of an HP 6890A GC [Hewlett-Packard (HP), Palo Alto, CA].

Gas chromatography (GC)/mass (MS) analysis and GC/FID quantification

SPME is a rapid, sensitive technique without using a solvent for the extraction of analytes from samples; it is carried via the automatized fiber injection system coupled to chromatographic separation instrument for the extraction of volatile and semivolatile organic compounds. SPME-trapped volatile components were identified by GC (Agilent HP 6890, Agilent Technologies, Palo Alto, CA) coupled to a mass spectrometer (Agilent HP 5973, Agilent Technologies) or a flame ionization detector. The injected sample was separated on a 5% diphenyl/95% dimethylpolysiloxan capillary column (30 m length, 0.25 mm inner diameter, and 0.25 µm film) (J&W Scientific, Santa Clara, CA). The gas chromatograph was programmed at a rate of 12°C/minute to 180°C, kept at 180°C for 5 minutes, operated under the following conditions: injector 220°C, column oven 40°C for 3 minutes, and finally ramped at a rate of 40°C/minute to 250°C and kept for 2 minutes and He carrier gas at 1 ml/minute. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV and a source temperature of 180°C. Volatile components were identified using the procedure

described in Farag and Wessjohann (2012) and peaks were identified by its retention indices relative to n-alkanes (C6–C20), mass spectrum matching to National Institute of Standards and Technology, and Wiley library database as well as published literature data.

Preliminary phytochemical screening

Sample of *A. cunninghamii* Aiton ex D. Don leaves was air-dried and ground to powder. The powdered sample was macerated in ethyl alcohol 70% for 3 days and then allowed for filtration and screened for the presence of the main classes of secondary metabolites such as flavonoids, tannins (Evans, 2002), sterols (Karnick, 1971), triterpenoids (Harborne and Baxter, 1999), saponins, cardiac glycosides, alkaloids (Fulton, 1937), anthraquinones (Fairbairn, 1949), and the presence of cyanogenic glycosides which were examined (Odugbemi, 2008) according to the approved methods of preliminary phytochemical tests.

Evaluation of antimicrobial activity

The potential antimicrobial activities of the methanolic extract of the plant species were studied through the determination of the zones of inhibition by agar well method (Cooper, 1972) against two standard Gram-positive bacteria, *Bacillus subtilis* ATCC-6633 and *Staphylococcus aureus* ATCC-6538, and two standard Gram-negative bacteria, *Pseudomonas aeruginosa* National Collection of Industrial Bacteria (NCIB) ATCC-9027 and *Escherichia coli* ATCC-8739, and the fungal strains were *Candida albicans* ATCC-90028 and *Aspergillus niger* ATCC-7966. The culture media used for bacteria were nutrient agar medium and malt extract agar medium used for cultivation of *C. albicans* and Czapek's Dox agar medium was used for *A. niger*. The strains were first checked for purity based on standard microbiological tests and then used for their sensitivity on the test samples. The bacteria and fungi were maintained on nutrient agar medium and Czapek's Dox agar medium, respectively. The agar media were inoculated with different test microorganisms. 100 and 200 µl of each methanolic plant extract diluted with 100 µl of dimethylformamide and the solubilized extract were placed in the well. After 24 hours of incubation at 30°C for bacteria and 48 hours of incubation at 28°C for fungi, inhibition zones were measured to the nearest mm using Vernier calipers. Standards with the concentration of 1 mg/ml were used as positive controls chloramphenicol (SEDICO Co., Egypt) for Gram-positive, cephalexin monohydrate [The Egyptian Co. for Pharmaceutical & Chemical Industries (EPCI), Egypt] for Gram-negative bacteria, and fluconazole (SEDICO Co., Egypt) for fungi.

Estimation of total phenolics content by Folin–Ciocalteu method

10 mg of each lyophilized methanolic extract of *A. cunninghamii* was dissolved in 10 ml 70:30 water/methanol (samples). 1 ml of each sample (1 mg/ml) was mixed with 1.5 ml of Folin–Ciocalteu; after 5 minutes, 4 ml of 20% sodium carbonate solution was added to make up to 25 ml with distilled

Table 1. Relative percentage of volatile components in *A. cunninghamii* using SPME/GC/MS.

No.	RT (minute)	KI	Ident. components	Area	Area %	Mol. Formula	Identification
1.	7.508	901	Tricyclene	5,297	5.21	C ₁₀ H ₁₆	MS, RI
2.	7.739	913	(-)- α -Pinene	33,522	32.95	C ₁₀ H ₁₆	MS, RI
3.	8.075	933	camphene	1,107	1.09	C ₁₀ H ₁₆	MS, RI
4.	8.5	957	Sabinene	1,215	1.19	C ₁₀ H ₁₆	MS, RI
5.	8.608	963	β -Pinene	6,606	6.49	C ₁₀ H ₁₆	MS, RI
6.	8.769	973	β -Myrcene	964	0.95	C ₁₀ H ₁₆	MS, RI
7.	9.155	998	3-Carene	253	0.25	C ₁₀ H ₁₆	MS, RI
8.	9.4325	1,015	<i>o</i> -Cymene	243	0.24	C ₁₀ H ₁₄	MS, RI
9.	9.508	1,020	Limonene	4,683	4.60	C ₁₀ H ₁₄	MS, RI
10.	11.128	1,129	α -Campholenal	219	0.22	C ₁₀ H ₁₆ O	MS, RI
11.	11.46	1,152	Isopulegol	422	0.41	C ₁₀ H ₁₈ O	MS, RI
12.	11.55	1,159	cis-Menthone	6,197	6.09	C ₁₀ H ₁₈ O	MS, RI
13.	11.746	1,172	d-Menthol	2,277	2.24	C ₁₀ H ₂₀ O	MS, RI
14.	11.881	1,183	p-Menthan-1-ol	25,451	25.02	C ₁₀ H ₂₀ O	MS, RI
15.	12.031	1,194	Isoneomenthol	525	0.52	C ₁₀ H ₂₀ O	MS, RI
16.	12.15	1,203	α -Terpineol	682	0.67	C ₁₀ H ₁₈ O	MS, RI
17.	12.488	1,229	cis-3-Hexenyl N-valerate	546	0.54	C ₁₁ H ₂₀ O ₂	MS, RI
18.	12.744	1,248	Pulegone	1,603	1.58	C ₁₀ H ₁₆ O	MS, RI
19.	13.008	1,269	Piperitone	398	0.39	C ₁₀ H ₁₆ O	MS, RI
20.	13.275	1,290	Isomenthol acetate	4,721	4.64	C ₁₂ H ₂₂ O ₂	MS, RI
21.	13.417	1,301	Anethole	302	0.30	C ₁₀ H ₁₂ O	MS, RI
22.	14.28	1,372	Eugenol	280	0.28	C ₁₀ H ₁₂ O ₂	MS, RI
23.	14.42	1,385	Copaene	814	0.80	C ₁₅ H ₂₄	MS, RI
24.	14.563	1,396	β -Elemene	216	0.21	C ₁₅ H ₂₄	MS, RI
25.	15.033	1,434	Caryophyllene	1,537	1.51	C ₁₅ H ₂₄	MS, RI
26.	15.283	1,455	Isocaryophyllene	217	0.21	C ₁₅ H ₂₄	MS, RI
27.	15.5	1,472	Humulene	196	0.19	C ₁₅ H ₂₄	MS, RI
28.	15.96	1,504	(+)-Ledene	218	0.21	C ₁₅ H ₂₄	MS, RI
29.	16.04	1,509	γ -Elemene	216	0.21	C ₁₅ H ₂₄	MS, RI
30.	16.27	1,526	δ -Cadinene	206	0.20	C ₁₅ H ₂₄	MS, RI
31.	22.76	1,877	(+)-Stachene	605	0.59	C ₂₀ H ₃₂	MS, RI
				10,1738	100.00		
				0	8		
			Monoterpenoids	96,142	94.51		
			Hydrocarbons	53,647	52.73		
			Oxygenated compounds	42,495	41.78		
			Sesquiterpenoids	3,620	3.54		
			Hydrocarbons	3,620	3.54		
			Oxygenated compounds	0	0		
			Tetracyclic diterpene	605	0.59		
			Hydrocarbons	605	0.59		
			Oxygenated	0	0		
			Phenyl propanoid	825	0.58		
			Fatty acid ester	546	0.54		

RT: Compound list in order of elution from column according to their retention time; KI: Kovat's index calculated on a polar column; Ident.: Identified; Mol.: Molecular.

water and was incubated for 30 minutes at room temperature, with absorbance at 765 nm. Blank was prepared in a similar way using 1 ml of 70:30 water/methanol instead of the sample.

A standard calibration curve was prepared by weighing accurately 25 mg of standard gallic acid and dissolving in 25 ml distilled water in a volumetric flask (stock solution 1 mg/ml) and then was diluted to (80–280) µg/ml; calibration was prepared by adding 1 ml of each dilution and then 10 ml of distilled water and 1.5 ml of Folin–Ciocalteu reagent were added; after 5 minutes, 4 ml of 20% sodium carbonate solution was added to make up to 25 ml with distilled water and was incubated for 30 minutes at room temperature, with absorbance at 765 nm (Saboo *et al.*, 2010).

All determination was performed in triplicate; the total content percentage was calculated as gallic acid equivalent (GAE).

$$\text{GAE} = [(C \times V)/M] \times 100$$

where *C* = the conc. of gallic acid established from calibration curve mg/ml, *V* = volume of extract (ml), and *M* = the weight of dried plant extract (mg).

Evaluation of total flavonoids

10 mg of each lyophilized methanolic extract of *A. cunninghamii* was dissolved in 10 ml 70% methanol (samples stock). 1 ml of each sample (1 mg/ml) was added separately to 1.5 ml methanol, 0.1 ml aluminum chloride, 0.1 ml potassium acetate solution, and 2.8 ml distilled water which were added and mixed well for each sample; the absorbance was recorded after 30 minutes at 415 nm. Blank was prepared in a similar way using methanol instead of the sample. Each sample was done in triplicate (Saboo *et al.*, 2010).

10 mg quercetin was dissolved in 10 ml methanol and then diluted to 5–100 µg/ml; to each dilution, the following reagents were added: 0.1 ml 10% AlCl₃, 0.1 ml of 1 M potassium acetate, and 2.8 ml distilled water. A calibration curve was prepared by measuring the absorbance of the different dilutions at 415 nm.

RESULTS AND DISCUSSION

Analysis of volatile constituents

The SPME technique followed by GC/MS analysis of *Araucaria* aerial parts sample resulted in the identification of 31 different volatile constituents. Monoterpenoids content was predominant representing 94.51%, among which hydrocarbon was 52.73% followed by oxygenated monoterpenoids constituting 41.78%. Sesquiterpenoids hydrocarbon reached only 3.54% with no oxygenated sesquiterpenes. (+)-Stachene was the only tetracyclic diterpene with 0.59%. Phenylpropanoids represent 0.82%. *cis*-3-Hexenyl N-valerate was the only fatty acid detected with 0.54%. Major compounds detected were the monoterpene hydrocarbons (–)-α-Pinene representing 32.95% followed by oxygenated monoterpene *p*-menthan-1-ol with 25.02%, listed according to their retention time in Table 1, and the gas chromatogram is shown in Figure 1.

Preliminary phytochemical screening

Preliminary phytochemical screening of *A. cunninghamii* Aiton ex D. Don leaves was carried out to investigate the phytochemical composition of the methanolic extract of the plant. Results showed that it contains phenolic compounds, flavonoids, and saponins. While cardiac glycosides, cyanogenic glycosides, alkaloids, and anthraquinones were absent, results are tabulated in Table 2.

Antimicrobial evaluation

Methanolic extract in both concentrations 100 and 200 µg/ml showed that *A. cunninghamii* showed moderate activity against G+ve *B. subtilis* ATCC-6633 and *S. aureus* ATCC-6538, Table 3, which increases by using the 200 µg/ml, and showed moderate activity against G–ve *P. aeruginosa* NCIB ATCC-9027 and *E. coli* ATCC-8739 using the 200 µg/ml concentration only. The methanolic extract of the species did not exhibit any activity against *A. niger* ATCC-7966 fungi or against the unicellular *C. albicans* ATCC-90028 as shown in Table 3.

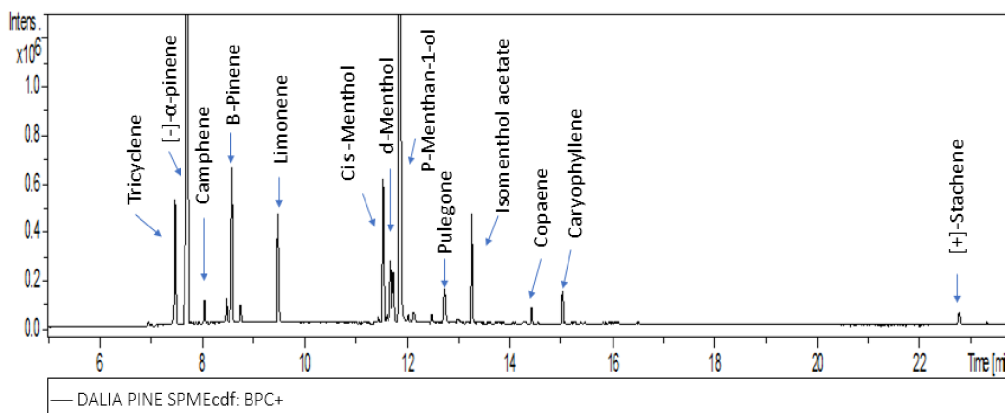


Figure 1. GC/MS total ion chromatogram of major volatile constituents of *A. cunninghamii* aerial parts.

Table 2. Results of phytochemical screening of *A. cunninghamii* aerial parts.

Constituents	<i>A. cunninghamii</i>
Carbohydrates and/or glycosides	+ve
Hydrolysable tannins	-ve
Condensed tannins	-ve
Free flavonoids	+ve
Combined flavonoids	+ve
Sterols and/or triterpenes	+ve
Alkaloids and/or nitrogenous bases	-ve
Cardiac glycosides	-ve
Anthraquinones	-ve
Cyanogenic glycosides	-ve

+ve: Present; -ve: Absent.

Table 3. Antimicrobial activity studies showing mean diameter of inhibition zones of various concentrations of *A. cunninghamii*.

Test organism	<i>A. cunninghamii</i> extract		Standards
	100 (mg/ml)	200 (mg/ml)	
<i>B. subtilis</i> ATCC-6633	15	17.5	31.5 ^a
<i>S. aureus</i> ATCC-6538	11	17	30 ^a
<i>P. aeruginosa</i> ATCC-9027	-ve	14	32 ^b
<i>E. coli</i> ATCC-8739	-ve	12	37.5 ^b
<i>C. albicans</i> ATCC-90028	-ve	-ve	25.5 ^c
<i>A. niger</i> ATCC-7966	-ve	-ve	22.5 ^c

+ve: Present; -ve: negative.

^a Standard chloramphenicol.^b Standard cephalixin monohydrate.^c Standard fluconazole; well diameter 1 cm (100 µl of each concentration was tested).

Total polyphenols and total flavonoidal content

From the following data, the estimated total polyphenolic content of the tested species was found that *A. cunninghamii* showed a high total polyphenolic content with 252.11 ± 0.28 mg/GAE, whereas it exhibited a high flavonoidal content of 21.65 ± 0.01 mg/µg compared to the standard quercetin.

Statistical analysis

All determinations were carried out in a triplicate manner and values are expressed as the mean \pm SD. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's test. Results are expressed as means \pm SEM ($n = 10$).

CONCLUSION

Volatiles were extracted from *A. cunninghamii* leaves using the headspace technique and analyzed by GC/MS. A total of 31 compounds were detected. (-)- α -Pinene was the major monoterpene hydrocarbon representing a third of the volatile constituents of the sample 32.95% and this is concurrent with

the plant being one of the conifers, followed by the menthane monoterpene p-menthan-1-ol representing 25.02%, and this is the first report on *A. cunninghamii* volatile constituents. The antimicrobial evaluation of the methanolic extract of *Araucaria* against multidrug-resistant pathogens using standard microbiological techniques by agar well diffusion method for activity against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. niger* isolated from clinical samples. The susceptibility patterns of the test isolates against the crude extract were determined at extract concentrations of 100 and 200 mg/ml, respectively. The results revealed that the extracts inhibit the growth of the tested G+ve spp. with a moderate extent followed by the G-ve spp. showing no activity for the fungus. The phytochemical screening revealed the presence of flavonoids-free and combined terpenoids and saponins. The estimated total polyphenolic content of *A. cunninghamii* showed a high total polyphenolic content with 252.11 ± 0.28 mg/GAE and exhibited also a high flavonoidal content of 21.65 ± 0.01 mg/µg compared to the standard quercetin. Finally, we need to save these precious species from logging to keep the natural ecosystem away from human's danger.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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