Doi: https://doi.org/10.17584/rcch.2024v18i1.16885

Antifungal activity of *Peperomia subspathulata* Yunck against three phytopathogenic fungi: *Aspergillus*, *Botrytis*, and *Penicillium* species

Actividad antifúngica de *Peperomia subspathulata* Yunck frente a tres hongos fitopatógenos: *Aspergillus*, *Botrytis* y *Penicillium*

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***Peperomia subspathulata* Yunk. In agroecological farm, Jardín Botánico José Celestino Mutis.**

Photo: D. Gutiérrez

ABSTRACT

Peperomia subspathulata Yunck is an aromatic plant of the Piperaceae family. This species is native to Colombia and Ecuador. It is used in food preparation for its aroma and has been traditionally used to treat blows and wounds. The present study evaluated the essential oil and the ethanolic extract of aerial parts from *P. subspathulata* against *Aspergillus* sp., *Botrytis* sp., and *Penicillium* sp. The essential oil was obtained by hydrodistillation, and gas chromatography coupled with mass spectrometry analyzed its composition. The ethanolic extract was obtained by maceration with ethanol 96%. The antifungal activity tests were conducted in a potato dextrose agar medium which different concentration of essential oil and extract were added. The main components identified in the essential oil were safrole (44.3%), α-bisabolol (24.2%), myristicin (4.7%), *trans*-β-caryophyllene (3.0%), viridiflorene (30%), α-humulene (2.3%), *trans*-nerolidol (1.5%), linalool (1.1%), methyleugenol (1.1%) and *cis*-farnesene (1.0%). The total phenolic content of the ethanolic extract was determined by the Folin-Ciocalteu method (48.5±0.5 mg gallic acid equivalent GAE/g of extract). The ethanolic extract and the essential oil inhibited the growth of microorganisms. The essential oil was the most effective against *Botrytis* sp. (MIC=500 μg mL^{-1}) and *Aspergillus* (MIC=1,000 μg mL^{-1}).

Additional key words: phytopathogenic fungi; essential oil; ethanolic extract; medicinal plants; bio-assays.

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RESUMEN

Peperomia subspathulata Yunck es una planta aromática de la familia Piperaceae. Es una especie nativa de Colombia y Ecuador. Se usa en la preparación de alimentos por su aroma y ha sido usada tradicionalmente para tratar golpes y heridas. En el presente estudio se evaluó el aceite esencial y el extracto etanólico de las partes aéreas de *P. subspathulata* frente a *Aspergillus* sp., *Botrytis* sp. y *Penicillium* sp. El aceite esencial fue obtenido por hidrodestilación y su composición fue analizada por cromatografía de gases acoplada a espectrometría de masas. El extracto etanólico se obtuvo por maceración con etanol al 96%. Los ensayos de actividad antifúngica fueron realizados en un medio de agar dextrosa papa al cual se le adicionaron diferentes concentraciones del aceite esencial o extracto. Los compuestos mayoritarios identificados en el aceite esencial fueron safrol (44,3%), α-bisabolol (24,2%), miristicina (4,7%), *trans*-β-cariofileno (3,0%), viridifloreno (3,0%), α-humuleno (2,3%), *trans*-nerolidol (1.5%), linalol (1,1%), metileugenol (1,1%) y *cis*-farneseno (1,0%). El contenido de fenoles totales del extracto etanólico se determinó por el método de Folin-Ciocalteu (48,5±0,5 mg EAG/g de extracto). El extracto etanólico y el aceite esencial inhibieron el crecimiento de los microorganismos. El aceite esencial fue el más efectivo frente a *Botrytis* sp. (CMI=500 μg mL^{-1}) y *Aspergillus* (CMI=1.000 μg mL^{-1}).

Palabras clave adicionales: hongos fitopatógenos; aceite esencial; extracto etanólico; plantas medicinales; bioensayos.

Received: 24-11-2023 Accepted: 06-03-2024 Published: 11-04-2024

INTRODUCTION

Phytopathogenic fungi are one of the main causes of losses in crop production. In the control of these pathogens mainly chemical fungicides are used, which, although effective, contaminate the environment and can remain on the treated leaves and fruits (Peng *et al.*, 2021). In the search for more environmentally friendly alternatives to control fungal diseases, fungicides of natural origin have emerged. Among the advantages of natural products are that they are less toxic because they do not bioaccumulate and are biodegradable (Sil *et al.*, 2020). The phytochemical compounds showed antibacterial, antiviral, antifungal, and insecticidal properties. For example, essential oils have shown the ability to control the growth of bacteria and fungi, as well as having insecticidal properties against flies, weevils, etc. (Sil *et al.*, 2020).

The *Peperomia* genus is known for traditional medicinal uses (García-Barriga, 1974; Valero Gutiérrez *et al.*, 2016) and *in vitro*, studies showed activity against bacteria, fungi, and yeast (Khan and Omoloso, 2002; Akinnibosun *et al*., 2008; Oloyede *et al.*, 2011). The essential oil of *Peperomia pellucida* showed bactericidal activity against *Staphylococcus aureus* (0.20 mg mL^{-1}) and *Listeria ivanovii* (0.15 mg mL^{-1}) (Okoh *et al*., 2017). Extracts of *Peperomia vulcanica* and *Peperomia fernandopoioana* showed moderate activity against *Escherichia coli* and *Staphylococcus aureus* and the essential oil and crude extract in methanol of *Peperomia galoides* were evaluated against *S. aureus* and *Staphylococcus epidermidis* showing total inhibition (Valero Gutiérrez *et al.*, 2016). Moreover, bioactive compounds have been identified from *Peperomia* species: grifolin and grifolic acid were isolated from *P. galoides* which moderately inhibited the growth of *S. aureus* and *S. epidermidis* (Langfield *et al.*, 2004); Patulosido A was isolated from essential oil of *P. pellucida* and was active against Gram-positive and Gram-negative bacteria (Khan *et al*., 2010). Two polyketides (2-acylcyclohexane-1,3-diones) with fungicidal properties against *Cladosporium cladosporioides* and *C. sphaerospermum* were isolated from *Peperomia alata* (Ferreira *et al*., 2014). *P. pellucida* showed activity against *S. aureus* (Oloyede *et al*., 2011) and its essential oil was active on *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Fusarium oxysporum* and *Aspergillus tamari* (Ogunmoye *et al.*, 2018).

P. subspathulata Yunck is native to Colombia and Ecuador (Ulloa Ulloa *et al.*, 2017), known as *canelo*, *canelon* and *siempre viva* (Jardín Botánico de Bogotá José Celestino Mutis, n.d.). It is used in food preparation and to treat blows and wounds (Angulo *et al*., 2012). Due to the limited knowledge of the chemical and biological activity of this species, the essential oil and ethanolic extract were evaluated against three

fungal organisms, and the chemical compounds of the essential oil were identified to establish another potential use of this aromatic species.

MATERIALS AND METHODS

Plant material

Plant material was collected from an agroecological farm at Jardín Botánico de Bogotá Jose Celestino Mutis. Carlos I. Suárez identified the collected specimen. The voucher specimen (CISB-700) was deposited in the Herbario of Jardín Botánico de Bogotá José Celestino Mutis.

Solvents and chemicals

Gallic acid (98%) was procured from Sigma–Aldrich Chemie (Steinheim, Germany). Ethanol (96%), dimethyl sulphoxide (DMSO), Folin–Ciocalteu's phenol reagent, anhydrous sodium sulfate, and sodium carbonate were purchased from Merck (Darmstadt, Germany). Spectrophotometric data were obtained on a Thermo Genesys model spectrophotometer and 1 cm pitch quartz cells were used.

Microorganisms

The strains of *Botrytis* sp. (SV-02), *Aspergillus* sp. (SV-97), and *Penicillium* sp. (SV-08) from the collection of the Bank of Jardín Botánico de Bogotá José Celestino Mutis were used for bioassays. The microorganisms were isolated from high Andean and Paramo plant species (*Espeletia barclayana* and *Quercus humboldtii*) and were reactivated on Potato Dextrosa Agar (PDA) medium and incubated at 27 °C.

Plant extracts

The essential oil (EO) and ethanolic extract were obtained from aerial parts of *P. subspathulata*. The EO was extracted from fresh material (990 g) by hydrodistillation for 3 h using a Clevenger-type apparatus. The EO was kept at 4°C with anhydrous sodium sulfate until further analysis (Elyemni, *et al.*, 2019). To prepare the ethanolic extract, the plant material was dried in an oven at 40°C (127.4 g) and submitted to maceration with ethanol (96%) for a week. The solvent was removed using a *Heidolph* rotary evaporator (Albarracin *et al.*, 2017).

Secondary metabolites of the ethanolic extract were screened qualitatively by using the methods of Soni and Sosa (2013) and Sanabria-Galindo *et al.* (1997). The presence of alkaloids (Dragendorff, Wagner, Mayer, Valser, and Hagger reagents), triterpenes and steroids (Salkowki, Liebermann-Buchard and Vanillin-phosphoric acid), flavonoids (Shinoda, Roseheim and leuco-anthocyans), quinones (acid and base test), phenols and tannins (ferric chloride and jelly-salt), and saponins (foam) were analyzed.

Total phenolic content

The total phenolic content of the ethanolic extract was determined by the Folin-Ciocalteu method (Singleton *et al*., 1999; Stanojević *et al*., 2009). Gallic acid was used as a standard, and the results were expressed as mg of gallic acid equivalents (GAE)/g of extract. The calibration curve was done using a dilution of gallic acid (5, 10, 25, 50,75, and 100 mg L^{-1}). 5 mL of Folin-Ciocalteu's reagent (dilution 1:10) was added to 1 mL of extract or standard and left to stand for 5 min. Then, 4 mL of a 7.5% sodium carbonate solution was added. The absorbance reading at 740 nm was taken 2 h later. The tests were performed in triplicate.

Characterization for essential oil

The relative density and refractive index of the EO obtained were determined by the pycnometer method at 20°C (ISO, 1998a), and the refractive index was measured by Abbe refractometer (ISO, 1998b). Gas chromatography–mass spectrometry (GC/MS) analysis was carried out on an Agilent Technologies 6890 gas chromatograph coupled to a mass selective detector (MSD, AT 5973N) in the electron impact mode (IE: 70 eV), operated in full scan mode with DB-5MS (60 m x 0.25 mm x 0.25 μm) and DBWAX (60 m x 0.25 mm x 0.25 μm) capillary columns. Injection was performed in Split mode (30:1), with an injection volume of 2 μL. The components were identified by the experimental determination of the linear retention indexes (LRI) and by comparing mass spectra with Adams, Wiley, and NIST databases (Stashenko *et al.*, 2008). The analyses were performed by the chromatography and mass spectrometry laboratory

(LABCROMASS) of the Universidad Industrial de Santander under contract JBB-CTO-864-2021.

Antifungal activity bioassays

The antifungal activity was determined using a mycelial radial growth inhibition technique (Sánchez-León *et al*., 2015). The assays were performed in 60 mm Petri dishes with a capacity of 5 mL of agar. 50 μL of each sample (EO, extract, or control) prepared in DMSO was added to a Petri dish, and agar PDA was added. Fungi with seven days of incubation at 27°C were used for the assays, an agar disc containing the microorganism was placed on agar with the test substance, and a control was included in each assay. Petri dishes were incubated at 27°C until the fungus of treatment reached the border of the dish. Petri dishes were checked daily until the fungus in the control covered the dish. With *Botrytis* the reading was done after 5 d. In the *Penicillium* sp. and *Aspergillus* sp. trials, the reading was done after 8 d, although they did not occupy the box completely. The analyses were performed in triplicate. Mycelial growth was determined by taking two measurements of the diameter (perpendicular) of the colony. The areas occupied by the colony were determined and the inhibition percentages were calculated with the following equation (1):

$$\%Inhibition = \frac{(C - T)}{C} \times 100 \quad (1)$$

where C corresponds to the radial growth of the mycelium in the control, and T to the radial growth of the mycelium in the treatment. The growth of the control will correspond to 100% because this corresponds to growth without inhibition. All tests were performed in triplicate. The minimal inhibitory concentration (MIC) of EO was defined as the lowest concentration of compound that completely inhibited visible growth after 5 and 8 d of incubation. And was determined by comparison with the control.

Statistical analysis

The data from the antifungal activity assays were evaluated using the Shapiro-Wilks test to establish whether they were normally distributed. Data that showed normal distribution were analyzed by Tukey's test for analysis of variance, and those that were not normally distributed were analyzed by the Kruskal Wallis test.

RESULTS AND DISCUSSION

Characterization of the essential oil

The EO was an oily-liquid, clear and, yellowish with a characteristic aroma of the plant and intense with fresh and cinnamon olfactory notes. The extraction yield of the EO was 0.16%, this was lower than the yield reported for fresh leaves and stems (1.0%) and roots (0.5%) (De Díaz *et al.*, 1988). The relative density determined was 1.0226 g mL^{-1} at 20°C and the refractive index at 20°C (n_D) was 1.5205. Upon observation of the EO, part of it remained on the water seal, while another portion remained in the lower part of the Clevenger trap, which means the essential oil is constituted by light compounds such as terpenes, and phenylpropanoids, that are denser than water. Moreover, the refractive index determined in this study was higher than that reported by De Díaz *et al.* (1988) of 1.3362. These variations may be due not only to the type of extraction but also to differences in plant materials, collection periods, and time of year, among other environmental factors. This significant difference suggests differences in the chemical composition of the EOs.

Characterization of the ethanolic extract

The ethanolic extract had a dark-colored, green-brown, liquid appearance with a sweet, herbal aroma less intense than the EO, and a yield of 34.5%. The total phenol content of the ethanolic extract was 1673±16 mg GAE/100 g dry material (48.5±0.5 mg GAE/g extract). This value had not been previously reported. However, its phenol content is close to that reported for *P. pellucida* extracted by maceration in butanol whose value was 42.73 (Phongtongpasuk and Poadang, 2014). The phytochemical analysis revealed the presence of phenolic compounds, triterpenes and steroids, alkaloids, flavonoids, quinones, and lactonic compounds. These results were consistent with the report of *Peperomia pellucida* (Oloyede *et al*., 2011) and *Peperomia blanda* except the presence of saponins (Al-Madhagi *et al*., 2019).

Chemical composition of essential oil

By gas chromatography analysis coupled with mass spectrometry, 95.4% of the compounds present in the EO were identified. The compounds were listed according to the elution order on the DB-5MS column

(Tab. 1). The calculated retention indices and those reported in the literature are included. The highest proportion of compounds were phenylpropanes (50.3%), followed by oxygenated sesquiterpenes (27.5%) and sesquiterpenes (13.6%). The main compounds identified were Safrole (44.3%), α-Bisabolol (24.2%), Myristicin (4.7%), trans-β-Caryophyllene (3.0%), Viridiflorene (3.0%), α-Humulene (2.3%), *trans*-Nerolidol (1.5%), Linalool (1.1%), Methyleugenol (1.1%) and *cis*-Farnesene (1.0%).

Table 1. Identified compounds from the essential oil of *Peperomia subspathulata*.

No	tR	LRI, DB-5MS		LRI, WAX		Identified compounds	Area (%)
		Exp.	Lit.	Exp.	Lit.		
1	20.3	1,034	1,026	1,214	1,211	1,8-Cineole	0.7
2	23.0	1,100	1,095	1,548	1,543	Linalool	1.1
3	26.9	1,199	1,190	1,702	1,694	α-Terpineol	0.4
4	30.6	1,301	1,287	1,883	1,872	Safrole	44.3
5	32.5	1,354	1,356	2,161	2,163	Eugenol	0.2
6	34.3	1,401	1,403	2,008	2,006	Methyleugenol	1.1
7	34.9	1,418	1,409	1,541	1,528	α-Gurjunene	0.1
8	35.4	1,432	1,419	1,610	1,599	*trans*-β-Caryophyllene	3.0
9	36.0	1,451	1,439	1,620	1,620	Aromadendrene	0.4
10	36.2	1,456	1,444	1,668	1,662	*cis*-β-Farnesene	1.0
11	36.6	1,468	1,452	1,667	1,689	α-Humulene	2.3
12	36.8	1,473	1,465	2,127	2,127	*trans*-Ethyl cinnamate	0.7
13	36.9	1,475	1,471	1,667	1,689	Acoradiene	0.3
14	37.2	1,485	-	-	-	$C_{15}H_{24}$	4.2
15	37.8	1,501	1,496	1,708	1,696	Viridiflorene	3.0
16	38.0	1,506	1,500	1,743	1,735	Byciclogermacrene	0.7
17	38.2	1,513	1,505	1,731	1,728	β-Bisabolene	0.2
18	38.2	1,515	1,514	-	-	β-Curcumene	0.4
19	38.6	1,528	1,519	2,262	2,261	Myristicin	4.7
20	38.7	1,530	1,521	1,777	1,771	β-Sesquiphellandrene	0.3
21	39.7	1,564	1,564	2,041	2,036	*trans*-Nerolidol	1.5
22	40.5	1,589	1,577	2,127	2,127	Spatulenol	0.3
23	40.7	1,595	1,582	1,990	1,986	Caryophyllene oxide	0.5
24	41.5	1,624	1,606	2,046	20,47	Humulene epoxide II	0.2
25	42.6	1,666	1,655	2,145	2,157	Bisabolol oxide II	0.3
26	42.7	1,670	-	-	-	$C_{15}H_{26}O_2$	0.5
27	42.9	1,675	1,669	2,606	-	Xanthoxylin	1.1
28	43.5	1,698	1,684	2,229	2,214	α-Bisabolol	24.2
29	49.7	1,973	-	-	-	$C_{18}H_{26}O$	1.9
30	50.6	2,016	-	-	-	$C_{18}H_{26}O$	0.4
Total percentage identified compounds							9.4

tR: retention time (min), LRI: linear retention index

According to the chemical composition of the EO, Safrole is the major compound with 44.3%, which is also reported by De Díaz *et al.* (1988) with 49%. Phenylpropanes are common compounds in *Peperomia* species, compounds such as safrole, myristicin and methyleugenol have been isolated from *Peperomia pellucida*, *Peperomia nitida*, *Peperomia campylotropa*, *Peperomia dindygulensis*, *Peperomia duclouxii*, *Peperomia oreophylla*, *Peperomia sui* and *Peperomia tetraphylla* (Valero Gutiérrez *et al*., 2016). The α-bisabolol is the second majority compound (24.2%) in the EO of *P. subspahulata*, this compound has also been identified in the EO of *P. galioides* (Villegas *et al*., 2001) and *P. sui* (Cheng *et al*., 2003).

Antifungal activity of ethanolic extract and essential oil

The ethanolic extract and the EO of *P. subspathulata* were evaluated against fungi at concentrations of 10, 100, and 1,000 μg mL^{-1} (Tab. 2). The data showed a normal distribution by the Shapiro-Wilks test (ethanolic extract against *Aspergillus* sp. and *Penicillium* sp.) and were analyzed for variance by Tukey's test. The results with a non-normal distribution were analyzed statistically by the Kruskal Wallis test.

The three fungi were susceptible to the treatments with the essential oil and the ethanolic extract. The best treatment with the EO was the concentration at 1,000 μg mL^{-1} as it inhibited the development of *Aspergillus*, *Botrytis*, and *Penicillium* by 94, 100, and 77%, respectively. In contrast, the ethanolic extract showed lower inhibition percentages than EO at 1,000 μg mL^{-1}, particularly, against *Aspergillus* sp. and *Botrytis* sp. *Aspergillus* sp. was the microorganism most susceptible to the extract (71.63%). While the percentage of inhibition against *Botrytis* was reduced by 43%.

The antifungal activity of the EO may be mainly correlated with the main compounds, phenylpropanoids, and terpenoids. Phenylpropanoids have shown remarkable antifungal properties. Isoeugenol was active against *Fusarium oxysporum* and *Botrytis cinerea* (Kfoury *et al.*, 2016), Eugenol against *Aspergillus niger* and *Penicillium digitatum* (Marei and Abdelgaleil, 2018). Valente *et al.* (2015) identified Myristicin as responsible for antifungal activity against *Aspergillus flavus* and *Aspergillus ochraceus*. Safrole showed activity against *Aspergillus fumigatus* (Poudel *et al.*, 2021). Moreover, a sesquiterpenoid α-Bisabolol was active against *Botrytis cinerea* (Kamatou and Viljoen, 2010), and *Aspergillus* species (Brandão *et al.*, 2020). The antifungal effect of the ethanolic extract could be due to the presence of flavonoid compounds, some authors associate these compounds with antifungal activity (Jin, 2019; Al Aboody *et al*., 2020; Di Ciaccio *et al*., 2020). Therefore, it is necessary to continue with chemical studies of the extract to identify the bioactive compounds.

The MIC determined that EO against *Aspergillus* sp. was 1,000 μg mL^{-1} and against *Botrytis* sp. 500 μg mL^{-1}. These results are very promising for the antifungal potential of this EO. Although there are no reports of other *Peperomia* species. However, Safrole pure was effective against *Aspergillus niger* and *Aspergillus fumigatus* with MIC of 78.1 and 39.1 μg mL^{-1}, respectively (Poudel *et al.*, 2021).

CONCLUSION

The essential oil of *P. subspathulata* is mainly constituted by phenylpropanes corresponding to 50.3%, followed by oxygenated sesquiterpenes and sesquiterpene hydrocarbons. The ethanolic extract and the essential oil showed activity against the

Table 2. Inhibition of fungal growth by essential oil and ethanolic extract of *Peperomia subspathulata*.

Concentration (μg mL^{-1})	Essential oil			Ethanolic extract		
	Aspergillus sp. (P=0.0036)	*Botrytis* sp. (P=0.0036)	*Penicillium* sp. (P=*0.0143*)	*Aspergillus* sp. (P=0.0003)	*Botrytis* sp. (P=0.0679)	*Penicillium* sp. (P=0.0012)
10	16.90±3.37	3.50±0.00	23.57±4.32	15.27±7.38 a	19.73±1.96	36.20±8.84 a
100	61.10±9.76	16.77±3.25	32.10±6.45	52.10±9.94 b	18.63±4.62	34.37±5.95 a
1,000	94.57±0.87	100.00±0.00	77.93±4.36	71.63±5.65 b	52.77±13.30	70.67±5.97 b
Control	11.64±3.74	13.85±2.78	11.98±4.16	10.28±1.64 a	7.47±1.67	14.20±3.07 a

Values represent means ± standard deviations for triplicate experiments. Different letters indicate significant differences (P<0.05) between concentration.

microorganisms evaluated; the essential oil was the most active against *Aspergillus* sp., *Botrytis* sp., and *Penicillium* sp. The results of this study are the first evidence of the antifungal potential of this plant, which is mainly used for its aroma in culinary preparations, and its effect against other microorganisms can be further explored to consider it as an alternative for agroecological management for the control of phytopathogenic fungi.

ACKNOWLEDGEMENTS

The author gratefully acknowledges funding from the Jardín Botánico José Celestino Mutis under-investment project 7679: Research for the conservation of the ecosystems and flora of the Region and Bogotá.

Conflict of interests: The manuscript was prepared and reviewed with the participation of the authors, who declare that there exists no conflict of interest that puts at risk the validity of the presented results.

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