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Chemical Composition and *in-vitro* biological activities of the essential oil from leaves of *Peperomia inaequalifolia* Ruiz & Pav.

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Abstract
Peperomia inaequalifolia Ruiz &Pav is a medicinal aromatic plant from Ecuador, known commonly as "congona", widely appreciated for its pharmacological attributes. The chemical study revealed the presence of 17 components, 14 of which were identified, such as safrole (32,10%), 11-αH-himachal-4-en-1-β-ol (25,29 %), myristicin(13,29 %), elemicin (10,07 %) and viridiflorol (5,24 %). The antiradical (DPPH test) and antioxidant (PCL) activity studies showed that the oil possesses an interesting activity, lightly lower than the natural reference, *Thymus vulgaris* essential oil. *The MIC results showed that the oil has very interesting antimicrobial and antifungal properties, particularly against* Gram+ bacteria (*Staphylococcus aureus*subsp. *Aureus* ATCC 6538 and *Streptococcus mutans*ATCC 25175) and two yeasts (*Candida tropicalis*ATCC 13803 and *Candida albicans*ATCC 10231).

Keywords: *Peperomia inaequalifolia*, essential oil, GC/MS, antioxidant activity, MIC.

1. Introduction
The genus *Peperomia* includes around 1000 species, distributed among the tropical and subtropical regions, mostly in Central and South America, only 17 in Africa [1], and widely spread in Ecuador where 224 species, 59 endemic [2], can be found. The species *Peperomia inaequalifolia* Ruiz &Pav is a native plant from the Piperaceae family [4], cultivated in the Andean region, between 1500 and 3500 meters above sea level, found mainly in the provinces of Azuay, Cañar, Carchi, and Chimborazo [2]. Traditionally, the plant is known as "congona", "cunguna", "cuncuna", or "trigresillo" [3].
From a medicinal point of view it has several properties, to be noted among them: analgesic [4, 5], antiparasitic [5], sedative [5], anti-osteoarthritic [5, 3], cardiac [4], hepatoprotective [4], and to treat sterility [4]. Because of its aromatic nature, it is used in Ecuador, in combination with other plants, to make a medicinal drink known as "horchata".

2. Materials and methods
2.1 Plant material
Peperomia inaequalifolia Ruiz & Pav species was collected on the Nayon site, Quito, Ecuador. The collection took place on the month of July of 2012. The National Herbarium of Ecuador was in charge of the botanical identification.

2.2 Isolation of essential oil using steam distillation vapors
Fresh *Peperomia inaequalifolia* leaves were distilled in a 250L-capacity distillation apparatus that belonged to the Chankuap Resources for the Future Foundation. The oil yield [% (w/w)] was calculated by taking into account the weight of the fresh material.

2.3 Gas Chromatography-Mass Spectrometry (GC-MS)
The essential oil was analyzed using GC-MS. The analysis was done with a Varian 3900 gas chromatograph equipped with a Factor Four column VF-5ms 5%-phenyl-95%-dimethylpolysiloxane (internal diameter of 30m × 0,25 mm, 0,25 µm film thickness) and directly interfaced to a Varian Saturn 2100 mass spectrometer. The carrier gas was helium (1mL/min) with a split ratio of 1:50. The oven temperature was initially 45 °C and then raised

to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min, and finally held at that temperature for 15 min. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1scan/min; mass range, 35-400 Da; trap temperature, 220 °C; transfer line temperature, 260 °C.

2.4 Determination of essential oil composition

The identification of compounds was achieved by comparing the EI-MS against commercial (NIST 2001) MS libraries and a homemade library. Additionally, the experimental calculation of retention indices was determined in relation to retention times from a series of *n*-alkanes (C10-C30); the theoretical calculation of retention indexes was compared to databases of aromatic molecules [6, 7].

2.5 Antioxidant properties

Radical-scavenging and antioxidant properties were analyzed through different assays, namely: 1,1-diphenyl-2-picrylhydrazyl (DPPH) spectrophotometric assay [8], and photochemiluminescence (PCL) [9].

2.4.1 Spectrophotometric DPPH assay

Several quantities of the *Peperomia inaequalifolia* Ruiz &Pav essential oil were dissolved with dimethyl sulfoxide (DMSO), up to a volume of 100 µl. We added 2,9 ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH; 1×10^{-4} in ethanol) to each solution, which was shaken vigorously and kept in the dark for 30 min at room temperature. Simple absorbance was measured at 517 nm with a UV-vis spectrophotometer (Shimadzu UV mini 1240). DMSO was used as a blank, while several solutions of essential oils from *Thymus vulgaris* and BHA were used for positive control.

The radical scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of DPPH:

$$Ip\ DPPH\% = \frac{Ab - Aa}{Ab} \times 100$$

Where Ab and Aa are the absorbance values of the blank sample and the test sample, respectively, after 30 min. Oils antiradical activity was considered as the concentration providing DPPH 50% inhibition (IC_{50}), calculated from inhibition curves obtained by plotting inhibition percentages against oil concentration.

2.4.2 Photochemiluminescence

Photochemiluminescence (PCL) measures the antioxidant capacity of pure substances or complex mixtures, both in the lipid (ACL) and water-soluble (ACW) phases. For measuring the antioxidant activity of the *Peperomia inaequalifolia* essential oil, we used the (ACL) method, most recommended for working on essential oils [10]. *Thymus vulgaris* was used for positive control and Trolox as a reference standard. The essential oils and reference standard were measured in the Photochem with the ACL kit (AnalytikJena, Jena, Germany).

2.5 Antibacterial and Antifungal Activity

For the evaluation of antimicrobial activity we used the disk diffusion method as described by several researchers of essential oils [11, 12, 13]. We used Gram-positive bacteria *Staphylococcus aureus*subsp. *aureus* ATCC 6538 and *Streptococcus mutans* ATCC 25175. We used Gram-negative bacteria *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027; and *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 10231 for yeasts. The antimicrobial activity can be described as a minimum inhibitory concentration (MIC), in mg/ml, using *Thymus vulgaris* essential oil as a natural reference.

3. Results & Discussion

3.1 Essential Oil Extraction

The essential oil yield obtained from the distillation of fresh leavesof *Peperomia inaequalifolia*, was 0,161 % (w/w).

3.2 Chemical Composition

The chemical composition of the essential oil evaluated with GC-MS, obtained the following results: the separation of 17 components, 14 of which were identified, as described on Table 1. The total number of components identified was equivalent to 93,33%, the most abundant components being: safrole, 11-αH-himachal-4-en-1-β-ol, myristicin, elemicin and viridiflorol.

Table 1: Essential Oil Composition of the *P. inaequalifolia* Leaves in %.

Components	RAA%	NI %	KI [a]	KI exp[b]
safrole	32,10		1287	1292
(*E*)-caryophyllene	2,22		1419	1420
γ-elemene	0,39		1436	1436
aromadendrene	0,25		1441	1442
α-humulene	0,37		1454	1459
germacrene D	0,37		1481	1484
viridiflorene	3,67		1496	1495
bicyclogermacrene	0,44		1500	1500
myristicin	13,29		1518	1520
elemicin	10,07		1557	1560
NI		0,79	-	-
spathulenol	0,44		1578	1589
N.I.		0,26	-	-
globulol	0,19		1590	1599
viridiflorol	5,24		1592	1598
11-αH-himachal-4-en-1-β-ol	25,29		1699	1708
NI		4,62	-.	-
Total identified	94,33			

[a]Kovats theoretical index, DB-5 column [14-15]. [b]Kovats experimental index calculated by comparing the retention rates of a homologous series of hydrocarbons C8-C30.

3.3 DPPH test

In order to evaluate the radical scavenging of the essential oil, we proceeded to calculate for each oil, the IC_{50} (concentration needed to inhibit oxidation by 50%), with both the DPPH. The results are shown on Table 2. We used *T. vulgaris* essential oil as a natural reference and the BHA as the reference molecule.

Table 2. IC50 for the *Peperomia inaequalifolia essential* oil via the DPPH

Essential oils	DPPH IC_{50} mg/ml
Peperomia inaequalifolia	2,220 ± 0,06
Thymus vulgaris	0,206 ± 0,01
BHA	0,059 ± 0,002

3.4 PCL test

The results of antioxidant activity, expressed in micromoles of Trolox equivalents per gram needed to inhibit the oxidation of luminol, can be observed on Table 3.

Table 3: Photochemiluminescence (PCL) of *Peperomia inaequalifolia essential* oil and reference essential oil *T. vulgaris*, Expressed as µmol of Trolox Equivalents/g.

Essential oils	µmolTrolox/g (P ≤ 0.05)
P. inaequalifolia	82,8
T. vulgaris	272.0

3.5 Antibacterial and Antifungal Activity

The results of the minimum inhibitory activity are shown on Table 4, with *T. vulgaris* essential oil as the positive control.

Table 4: Antibacterial and Antifungal Activity of the Essential Oils of *Peperomia. Inaequalifolia* leaves.

MICROORGANISMS	*P. inaequalifolia* MIC (mg/ml)	*T. vulgaris* MIC (mg/ml)
Gram-negative bacteria		
Escherichia coli ATCC 8739	0.77	0.39
Pseudomonas aeruginosa ATCC 9027	3.09	6.08
Gram-positive bacteria		
*Staphylococcus aureus*subsp. *aureus* ATCC 6538	0.10	1.54
Streptococcus mutans ATCC 25175	0.10	1.54
Yeasts		
Candida tropicalis ATCC 13803	0.39	0,77
Candida albicans ATCC 10231	0.39	0.39

The MIC results were very interesting for Gram+ bacteria, *P. aeruginosa* and the yeast *C. tropicalis*: the results for these microorganisms were higher than those determined for the positive control. The major component, safrole, has shown antibacterial [14] and anti-candical [15] activities, this would explain the good performance of the essential oil.

4. Conclusions

One of the main purpose of this investigation, that was possible to achieve, was showing the majority of essential oil components. It is a product that can be considered as the natural source of safrole, because a 32,10 % amount safrole was characteristic of the essential oil, with values that are similar to the ones presented by the *T. vulgaris* essential oil. This research presents high values of antimicrobial activity for the Gram + bacteria and for the yeast, higher than the ones present in the used oil as a reference.

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