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Short Communication

ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF HYDROALCOHOLIC EXTRACTS FROM *ALOYSIA POLYSTACHYA* GRISEB MOLDENKE AND *LIPPIA TURBINATA* GRISEB (VERBENACEAE)

MARÍA INÉS AGUADO[a*], NÉSTOR HUGO DUDIK[a], CRISTINA MARISEL PÉREZ ZAMORA[a,b], CAROLA ANALÍA TORRES[a,b], MARÍA BEATRIZ NUÑEZ[a]

[a]Department of Basic and Applied Sciences. Pharmacy Career, National University of Chaco Austral. P. R. Sáenz Peña, Chaco. Argentine, [b]National Scientific and Technical Research Council (Conicet). C. A. B. A. Argentine
Email: marynes@uncaus.edu.ar

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ABSTRACT

Objective: The aims of this study were to determine polyphenols and flavonoids composition, to evaluate the antioxidant and antibacterial activities and possible antibacterial synergistic effects of hydroalcoholic extracts of *Aloysia polystachya* and *Lippia turbinata.*

Methods: The flavonoids analysis was carried out in ethyl acetate fractions by means of high-performance liquid chromatography coupled to electrospray ionization quadrupole time of flight mass spectrometry (HPLC-ESI-Q-TOF-MS). The phenolic content was measured using Folin Ciocalteu reagent. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical method was used to estimate the antioxidant capacity. The antibacterial activity was determined by the agar microdilution and broth microdilution methods. The checkerboard assay was carried out to determine possible antibacterial synergism.

Results: Several flavonoid compounds were detected for the first time in these species, being *Lippia turbinata* extract the one that presented the highest total phenolic content and antioxidant activity. Extracts from both species would have bactericidal activity against *Staphylococcus* strains employed in this work. The extract combinations had a synergistic effect against three bacteria, one of them Gram-negative.

Conclusion: These extracts have demonstrated antioxidant and antibacterial activity, and its combination showed a synergistic effect against methicillin-resistant *Staphylococcus aureus*. Biological activities would be attributed to the whole of phytochemical constituents present in the respective crude extracts. However, it could be considered that the found flavonoids play an important role in the development of these activities.

Keywords: *Aloysia polystachya*, Antibacterial activity, Antioxidant activity, Flavonoids, *Lippia turbinata*

Aloysia polystachya (Griseb.) Moldenke and *Lippia turbinata* (Griseb.) belong to the Verbenaceae family and have a broad ethnomedicinal use in the Argentine. The aims of this study were to determine polyphenols and flavonoids composition, to evaluate the antioxidant and antibacterial activities and possible antibacterial synergistic effects of hydroalcoholic extracts of *A. polystachya* (ApE) and *L. turbinata* (LtE).

Plant material was collected at the campus of National University of Chaco Austral in December 2013, and March 2014. The extracts were prepared by hydroalcoholic simple percolation [1], with some modifications, from 60 g of dried leaf powder (particle size 840-1680 µm) and ethanol (EtOH) 70 °. The final volume (60 ml) of each extract was filtered under reduced pressure. Hexane was added, and the EtOH fraction was separated. Then, ethyl acetate (EtOAc) and water were added. Both fractions were evaporated to dryness, dissolved in HPLC quality methanol and filtered through 0.45 µm membrane.

The polyphenols study (in EtOAc fractions of the extracts) was carried out at the Higher Institute of Research, Development and Food Service (ISIDSA), National University of Córdoba, Argentine, by means of an HPLC-ESI-Q-TOF-MS analysis. Mass spectra were registered in the negative mode in the range of 100 to 1000 mass to charge ratio (*m/z*).

Total phenolic content was determined using Folin Ciocalteau reagent [2]. It was calculated from the standard curve of gallic acid solutions. Samples were analyzed in triplicate.

For the reduction of DPPH radical [3], fresh DPPH radical stock solutions and plant extract dilutions (1:10) were prepared in EtOH 70 °. Absorbance was measured at 517 nm. The DPPH inhibition percentage was calculated by means of the equation: [(Absorbance $_{control}$−Absorbance $_{sample}$)/Absorbance $_{control}$] x 100 and the results were expressed as Inhibitory Concentration 50 (IC_{50}). To determine the antioxidant activity, differences in absorbance were compared with antioxidants standards plots made from ascorbic acid and 6-hydroxy-25, 7, 8-tetramethyl-chroman-carboxylic acid (Trolox®) solutions. Results were expressed as Ascorbic Acid Equivalent Antioxidant Capacity (AAEAC) and as Trolox Equivalent Antioxidant Capacity (TEAC). These measurements were performed in triplicate.

For antibacterial assays, six reference strains and two clinical isolates were tested (provided by the Hospital Ramón Carrillo, from Sáenz Peña city, Chaco, Argentine). The extracts were dried and dissolved in dimethyl sulfoxide. Minimal inhibitory concentration (MIC) values of the extracts were determined by the serial agar macrodilution and the broth microdilution methods [4]. The microdilution method was also used to determine minimal bactericidal concentration (MBC) values. Ampicillin was used as a control.

The antimicrobial synergy between extract combinations was studied by the checkerboard assay. Then, the fractional inhibitory concentration (FIC) and the fractional inhibitory concentration index (FICI) were calculated [5].

All quantitative assays were made in triplicate and were expressed as the average of three analyses±Standard Deviation (SD).

Phenolic composition of the studied species is summarized in table 1. Flavones were predominant in both samples. Chrysoeriol and diosmetin are flavones with the same molecular weight and molecular formula, differing only in the position of the methoxy group (3´ or 4´). Mass spectra fragmentation is similar to both compounds. It was not possible to make a distinction between both flavones. In this sense, further studies are needed in order to identify these compounds.

Table 1: Retention times and MS data of phenolic compounds in ethyl acetate fractions

Ethyl acetate fractions	Retention time (min)	Parent ion (*m/z*)	Product ion (*m/z*)	Tentative identification
ApE, LtE	15.6	623	461	chrysoeriol diglycoside/ diosmetin diglycoside [b]
ApE, LtE	18.6	623	461	chrysoeriol diglycoside/ diosmetin diglycoside [b]
LtE	19.0	447	285, 225, 235	luteolin-O-glycoside [b]
LtE	19.2	461	285, 415, 233, 191, 327	luteolin-O-glucuronide [b]
LtE	19.9	477	433, 315, 300, 271	isorhamnetin-O-glycoside [b]
ApE, LtE	25.8	315	300	isorhamnetin [a]
ApE, LtE	25.9	285	175, 199, 217, 241	luteolin [b]
ApE, LtE	29.0	269	225	apigenin [b]
ApE, LtE	29.1	299	284	chrysoeriol/diosmetin [b]
ApE, LtE	31.1	313	298, 283	velutin [a]

[a]Confirmed with base data. [b]Confirmed with base data and references,

Table 2: Total phenol content and DPPH antioxidant activity

Substance	Phenolic content (mg GAE/ml E) [a*]	DPPH-IC_{50} (mg GAE/ml R) [b*]	DPPH-AAEAC (mg AAE/ml E) [c*]	DPPH-TEAC (mg TE/ml E) [d*]
ApE	4.29±0.10	$4.83x10^{-3}$±$1.63x10^{-4}$	4.18±0.12	1.69±$6.55x10^{-2}$
LtE	7.95±$4.75x10^{-3}$	$1.66x10^{-3}$±$1.29x10^{-5}$	22.85±0.52	3.44±$2.13x10^{-2}$
Ascorbic acid (0.36 mg/ml)	---	$4.72x10^{-3}$±$3.04x10^{-4}$	---	---
Trolox® (0.025 mg/ml)	---	$1.90x10^{-3}$±$9.80x10^{-5}$	---	---

[a]Milligram gallic acid equivalent per milliliter of extract. [b]Milligram gallic acid equivalent per milliliter of reaction. [c]Milligram ascorbic acid equivalent per milliliter of extract. [d]Milligram Trolox equivalent per milliliter of extract. [*]mean±SD (n=3), all values are significantly different ($p<0.05$, Student test).

Table 2 includes the total phenol content and results of antioxidant activity by DPPH method. The LtE presented the highest total phenolic content and antioxidant activity.

The results of bacterial susceptibility to extracts and interaction among them are represented in table 3. According to Noumeden *et al.* criterion [6], both extracts would have bactericidal activity against all *Staphylococcus* strains used in this work. The LtE showed a bactericidal effect against *E. faecalis* ATCC 29212 whereas ApE was bacteriostatic. In addition, LtE had a bacteriostatic effect on *E. coli.* When the extracts were combined, an additive effect against *E. faecalis* ATCC 29212 and *S. aureus* (ATCC 25923 and ATCC 29213) was observed, whereas a synergistic effect was observed against *S. epidermidis* ATCC 12228, *S. aureus* MRSA and *E. coli* ATCC 35218.

Table 3: Antibacterial activity of extracts

Strains	Ampicillin	Individual extracts		Extract combinations (synergy test)			
	MIC/MBC (µg/ml) [*]	MIC/MBC ApE (µg GAE/ml E) [*]	MIC/MBC LtE (µg GAE/ml E) [*]	MIC ApE/MIC LtE	FIC ApE	FIC LtE	FICI
E. faecalis ATCC 29212	0.4/1.6	250/> 1000	250/500	31.25/125	.13	0.50	0.63 [a]
S. aureus ATCC 25923	0.4/1.6	250/500	250/250	31.25/125	0.13	0.50	0.63 [a]
S. aureus ATCC 29213	0.8/1.6	250/500	250/250	62.5/125	0.25	0.50	0.75 [a]
S. aureus MRSA	25.6/102.4	250/500	250/500	62.5/62.5	0.25	0.25	0.50 [b]
S. epidermidis ATCC 12228	3.2/6.4	250/1000	125/250	62.5/3.90	0.25	0.03	0.28 [b]
E. cloacae (isolate)	204.8/614.4	> 1000/> 1000	> 1000/> 1000	---	---	---	---
E. coli ATCC 35218	204.8/614.4	> 1000	250/> 1000	500/31.2	0.25	0.13	0.38 [b]
P. aeruginosa ATCC 27853	ND	> 1000	1000/> 1000	---	---	---	---

FIC: Fractional Inhibitory Concentration. FICI: Fractional Inhibitory Concentration Index. [a]Additivity. [b]Synergy. ND: not detected in the range of tested concentrations (0.05-614.4 µg/ml). [*]Mean values, n=3.

In previous studies, we detected mainly phenols/tannins, flavonoids, and terpenes in these Verbenaceae. We also found phenols and flavonoids as well as antioxidant activity in another *A. polystachya* ethanolic extract [7, 8]. Moreover, we recognized the presence of flavonoids (probably flavonols and flavones) in ethanolic LtE [8-10]. Our actual findings reinforce the fact that the main identified flavonoids in *Lippia* genus were flavones [11]. The secondary metabolites found in these species could provide a preliminary explanation on their activities. The phenolic content is an important factor for the antioxidant capacities of the plants, and many of the antioxidant compounds also exhibit antimicrobial action [12, 13]. Flavonoids have been described to possess antibacterial action even against resistant bacteria [14].

The qualitative flavonoid composition of both extracts was quite similar, differing LtE by the presence of two derivatives of luteolin and an isorhamnetin derivative. In this opportunity, we found flavones (apigenin and probably chrysoeriol) in LtE coinciding with a preliminary characterization of the major flavonoids from methanolic extracts from Tucumán province [15].

The bactericidal activity of both extracts against *S. aureus* ATCC 25923 found herein is complementary to Toribio *et al.* report [16]. Our results are partly consistent with other works in which flavonoids found in methanolic LtE showed antimicrobial activity [15, 17-19].

It is known that apigenin has a moderate antibacterial activity against *P. aeruginosa* ATCC 27853. Additionally, luteolin has significant activity against *E. coli*, *P. aeruginosa* and *S. aureus* while compounds derived from luteolin and apigenin show not only free radical scavenging activity but also antimicrobial activity against *E.*

coli [20-24]. Some researchers have reported several flavonoids that possess antimicrobial activities, including isorhamnetin and their derivatives. They have discovered that diosmetin, luteolin, luteolin glycoside and isorhamnetin glycoside demonstrated activity against *S. aureus* ATCC 6538, *E. cloacae* human isolate, *E. coli* ATCC 35210, and *P. aeruginosa* ATCC 27853 [23, 24].

An antibacterial effect of each extract and a synergistic effect in combination might be an interesting alternative therapy for infectious diseases caused by MRSA strains and some Gram-negative bacteria as *E. coli* ATCC 35218. Since the determinations in this work were performed with the total hydroalcoholic extract of each species, the assessed biological activities could be attributed to the whole phytoconstituents. However, it could be considered that the found flavonoids play an important role in the development of these activities. To our knowledge, this is the first time that apigenin is reported in *A. polystachya*. In the same way**,** luteolin, isorhamnetin, and velutin are reported for the first time in these two Verbenaceae. Further work is essential on the isolation and identification of more bioactive components from their extracts.

CONFLICT OF INTERESTS

All authors have none to declare

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