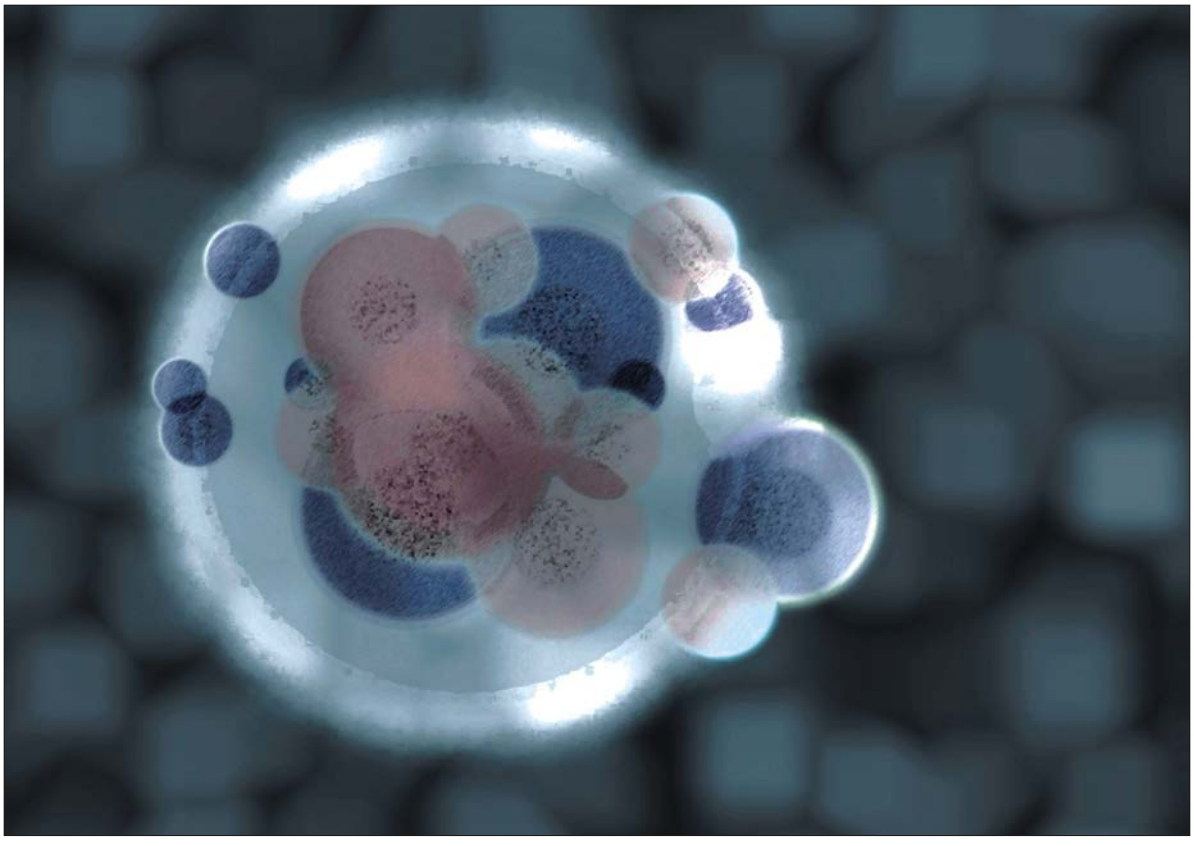

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Antimicrobial Activity of the Leaves of *Lippia triphylla* (L'Her) O. Kuntze (Verbenaceae) Against on Bacteria and Yeasts

Mustafa Oskay, A. Üsame Tamer, Güngör Ay, Dilek Sari and Kàmuran Aktaş
Department of Biology, Faculty of Sciences and Arts,
Celal Bayar University, Campus of Muradiye, 45030, Manisa, Turkey

Abstract: Herbal medicine represents one of the most important fields of traditional medicine in Turkey, especially in rural areas. Thus, phytotherapy is practiced by a large proportion of Turkey population for the treatment of several physical, physiological, mental and social ailments. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs it is essential to study medicinal plants, which have folklore reputation in a more intensified way. The crude ethanolic extract of the leaves of *Lippia triphylla* showed varying degrees of antimicrobial activity against on nine pathogenic bacteria and four yeasts by the agar-well diffusion method. The Minimal Inhibitory Concentration (MIC) values for *S. aureus, K. pneumoniae*, *P. vulgaris* and *C. albicans* were 10, 30, 22 and 6 mg mL^{-1}, respectively. The antimicrobial activity related to standard antibiotics was determined as positive control and comparison. Minimal Bactericidal Concentration (MBC) values ranging from 10 to 50 mg mL^{-1}.

Key words: *Lippia triphylla*, lemon verbena, verbenaceae, antimicrobial activity

INTRODUCTION

Various medical plants have been used for years in daily life to treat disease all over the world. Turkey is an important floristic center internationally because of its geographic location, climate and the presence of nearly 10,000 natural plant species[1]. Within the recent years, infections have increased to a great extent and antibiotics resistance becomes an ever-increasing therapeutic problem. Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action.

Lippia triphylla (L'Her) O. Kuntze [Syn: *Aloysia triphylla* (L'Her.) Britt., Fam. Verbenaceae] is a scented, perennial herb with several stiffly up to 2.5 m erect stems, lanceolate leaves, till 10 cm, in verticals of 3, petiolated, flowers gathered in racemes, bell-shaped, purplish outside, more whitish inside, which can be found in West Asia, North Africa and throughout Europe[2,3]. The leaves of this species are reported to possess antidepressant, antispasmodic, antipyretic, sedative and anticonvulsant effect as well as its use for the treatment of jaundice and digestive problem[2-5]. It has also been used in Turkey as a folk medicinal herbal tea for coughs.

Previous studies on *L. triphylla* concerned its chemical characterization and revealed the presence of several flavonoids and phenolic acids, such as limonene, neral, geranial, geranyl acetate, pinene, sabinene, 1,8-cineol, trans -β-ocimene, β-caryophyllene, germacrene D and farnesene[4-8].

The present study reports the first attempt to study the antimicrobial activity of the crude extract of *L. triphylla* leaves of the Turkey using both diffusion techniques on solid media and macrobroth dilution methods in broth media.

MATERIALS AND METHODS

Plant material: *L. triphylla* leaves were collected in Botanical Garden, Campus of Muradiye, Celal Bayar University, in August 2003 and identified by Dr. Güngör Ay. A voucher specimen was deposited at Department of Biology, CBU.

Microorganisms and growth conditions: Test microorganisms included the following bacteria: *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* CCM 2318, *Bacillus cereus* CM 99, *Escherichia coli* ATCC 11230, *Micrococcus luteus* ATCC 9341, *Pseudomonas fluorescens, Proteus vulgaris* ATCC 6997, *Salmonella typhimurium* CCM 5445, *Enterobacter faecalis* ATCC 29212 and for yeast *Candida albicans*,

Corresponding Author: Mustafa Oskay, Department of Biology, Faculty of Sciences and Arts, Celal Bayar University, Campus of Muradiye, 45030, Manisa, Turkey
Tel: +90 236 2412151-122 Fax: +90 236 2412158

Saccharomyces cerevisiae, *Yarrawia lipolytica*, *Geotrichum penicillatum*. Cultures of these bacteria were grown in Mueller-Hinton Broth (Oxoid) at 37°C for 24 h and the studied yeasts were incubated in Glucose Yeast Extract Broth at 30°C for 48 h. All the microorganisms were obtained from the Department of Biology, Ege University (İzmir/Turkey).

Preparation of the crude ethanolic extracts: The leaves of the plant material were dried and powdered finely using a blender. Powdered leaves (30 g) was extracted 150 mL 96% (w/w) ethanol for 16 h by using a Soxhlet extractor apparatus. The extract was filtered using whatman filter paper No. 4 and the filtrate was then evaporated under reduced pressure and dried at 45°C. The dried residue crude extract was resuspended in 96% ethanol at a concentration of 200 mg mL^{-1} and stored in labeled sterile screw capped bottles at +4°C for further experiments.

Antimicrobial assay

Agar well diffusion assays: The antimicrobial activity was determined by the agar-well diffusion method[9] with slight modification according to the present experimental conditions. Fifty microliter inoculum (10^8 bacteria per mL and 10^7 yeasts per mL) was added to 15 mL melted Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) medium cooled at 45°C. This was then poured into 9 cm. diameter petri dishes and maintained for 1 h at room temperature. Small wells (6 mm diameter) were cut in the agar plate using a cork borer. Extract concentration (10 and 20 μL) with a negative control (96% ethanol, 20 μL) were loaded in the wells. The dishes were then inverted and preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation, for bacteria, the plates were inverted and incubated aerobically at 37 and 28°C for 24 and 48 h, respectively for yeasts. The antimicrobial activity was evaluated by measuring the inhibition zone diameter observed. In addition, reference antibiotic discs such as penicillin G (10 i.u.), nalidixic acid (30 μg), novobiocin (30 μg), imipenem (10 μg), erythromycin (15 μg), chloramphenicol (30 μg) and nystatin (10 μg) were used as positive control and comparison.

Minimal Inhibitory Concentration (MIC) determination: Minimal inhibitory concentration was tested for bacteria in broth media Mueller-Hinton and yeasts in Glucose Yeast Extract Broth using the macrobroth dilution method[10]. In these experiments, 0.5 mL of a suspension containing $1x10^8$ cfu mL^{-1} was added to 4.5 mL of susceptibility test broth containing serial twofold dilutions of the extract in glass test tubes (16 by 100 mm) fitted with loose plastic nonscrew caps. All tubes were incubated in air at 37°C for 24 h before being read. The MIC was considered the lowest concentration of the sample that prevented visible growth. Minimum Bactericidal Concentrations (MBC) were determined by subculturing, 10 μL from each negative tube and from the positive growth control. MBC were defined as the lowest concentration yielding negative subcultures or only one colony. All samples were examined in duplicate in three separate experiments.

RESULTS AND DISCUSSION

In earlier studies, the antioxidant activity by Valentão *et al.*[3], the aromatic and polyphenolic composition by Carnat *et al.*[6] of *L. triphylla* leaves and also traditional uses, chemistry, antimicrobial activities and pharmacology of other *Lippia* species were reported[4,8,11]. Now the antimicrobial activity of the crude ethanolic extract of the *L. triphylla* leaves has been reported against some pathogenic Gram-positive, Gram-negative bacteria and yeasts. The zones of inhibition ranged from 2 to 22 mm (dose: 20 μL= 4 mg $well^{-1}$). The ethanolic extract of the leaves *L. triphylla* showed activity against all tested microorganisms except for *P. fluorescens* and *S. cerevisiae*. Maximum inhibitions were shown against on *S. aureus* and *C. albicans*, 16 and 22 mm, respectively. *C. albicans* had the lowest MIC of 6 mg mL^{-1} and the highest MIC was >50 mg mL^{-1} for *B. cereus*, *M. luteus* and *S. typhimurium*. The lowest MBC obtained with *L. triphylla* crude ethanolic extract was 10 mg mL^{-1} for *C. albicans*. MBC values for *S. aureus*, *K. pneumoniae* and *P. vulgaris* were 16, 50 and 26 mg mL^{-1}, respectively (Table 1).

The antimicrobial activity of this extract showed a weak variable degree against Gram-positive and

Table 1: Antimicrobial activity of the ethanolic extract of the leaves of *L. triphylla*

	Dose (μL)			MIC	MBC
Tested microorganisms	10[a]	20[b]	20[c]	(mg mL^{-1})	(mg mL^{-1})
S. aureus ATCC 6538P	8*	14[d]	0	10	16
K. pneumoniae CCM 2318	2	6	0	30	50
B. cereus CM 99	0	3	0	>50	ND
M. luteus ATCC 9341	0	3	0	>50	ND
E. coli ATCC 11230	0	2	0	ND	ND
P. fluorescens	0	0	0	ND	ND
S. typhimurium CCM 5445	0	3	0	>50	ND
E. faecalis ATCC 29212	0	3	0	ND	ND
P. vulgaris ATCC 6997	6	9	0	22	26
C. albicans	17	22	0	6	10
S. cerevisiae	0	0	0	ND	ND
Y. lipolytica	3	7	0	ND	ND
G. penicillatum	3	9	0	ND	ND

[a] 2 mg $well^{-1}$ (6 mm), [b]4 mg $well^{-1}$, [c]control, 20 μL 96% ethanol, * mean values, n=3, [d]inhibition zone diameter in mm, not including well diameter, 0 no inhibitory activity, ND not determined

Table 2: Inhibitory activity of the some standard antibiotics against various bacteria and yeasts in a disk diffusion assay

	Standard antibiotics						
Tested microorganisms*	NA[a]	P	NV	IPM	ERY	CHL	NYS
S. aureus ATCC 6538P	15[b]	36[c]	32	48	30	30	ND
K. pneumoniae CCM 2318	11[R]	0	0	21	10[R]	15	ND
B. cereus CM 99	14	10[R]	8[R]	24	20	25	ND
M. luteus ATCC 9341	0	15	20	0	12[R]	0	ND
E. coli ATCC 11230	22	0	0	30	12[R]	25	ND
P. fluorescens	16	0	6[R]	26	17	8[R]	ND
S. typhimurium CCM 5445	0	0	0	20	15	15	ND
E. faecalis ATCC 29212	ND	ND	ND	ND	ND	ND	ND
P. vulgaris ATCC 6997	20	0	0	25	15	22	ND
C. albicans	0	0	0	0	0	0	22
S. cerevisiae	0	0	0	0	0	0	20
Y. lipolytica	0	0	0	0	0	0	ND
G. penicillatum	0	0	22[±]	20[±]	0	16[±]	ND

* Bacteria tested in MHA medium, yeasts in PDA, [a] NA Nalidixic acid (30 μg disc^{-1}), P Penicilline G (10 i.u. disc^{-1}), NV Novobiocin (30 μg disc^{-1}), IPM Imipenem (10 μg disc^{-1}), ERY Erythromycin (15 μg disc^{-}), CHL Chloramphenicol (30 μg disc^{-}), [b]NYS Nystatin (10 μg disc^{-}), [b]Diameter of inhibition zone in mm, including disc diameter (6 mm), [c] mean values, n=3, [R] resistant, ±partially inhibition, 0 no activity, ND not determined

Gram-negative bacteria that resistant to broad spectrum antibiotics such as nalidixic acid, penicilline G, novobiocin, imipenem, erythromycin and chloramphenicol (Table 2).

The results of this investigation give support to the traditional use of the leaves of *L. triphylla* in the treatment of infectious diseases caused by *C. albicans* and some pathogenic bacteria such as *S. aureus*, *K. pneumoniae*, *E. coli* and *S. typhimurium*.

There is now preliminary scientific validation for the use of *L. triphylla* leaves for antibacterial and antifungal activity as a medicinal plant. The active phytocompounds of this plant used against multidrug-resistant bacteria and its toxicity have to be determined in further studies.

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