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Antimicrobial Activity of *Arctium lappa* Constituents Against Microorganisms Commonly Found in Endodontic Infections

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This study evaluated *in vitro* the antimicrobial activity of rough extracts from leaves of *Arctium lappa* and their phases. The following microorganisms, commonly found in the oral cavity, specifically in endodontic infections, were used: *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans*. The agar-diffusion method allowed detection of the hexanic phase as an inhibitor of microbial growth. Bioautographic assays identified antimicrobial substances in the extract. The results showed the existence, in the rough hexanic phase and in its fractions, of constituents that have retention factors (Rf) in three distinct zones, thereby suggesting the presence of active constituents with chemical structures of different polarities that exhibited specificity against the target microorganisms. It may be concluded that the *Arctium lappa* constituents exhibited a great microbial inhibition potential against the tested endodontic pathogens.

Key Words: Endodontics, antimicrobial activity, *Arctium lappa*.

INTRODUCTION

Arctium lappa is a plant brought from Japan and acclimated in Brazil, which is widely used in popular medicine all over the world for its well-known therapeutic applications. It has anti-bacterial and antifungal activity (1-3), diuretic (1,4), anti-oxidant (5) and anxiolytic (6) action, platelet anti-aggregating effect (7) and HIV-inhibitory action (8).

In Dentistry, *Arctium lappa* has been investigated due to its antimicrobial potential against oral microorganisms, specifically those associated with endodontic infections, as demonstrated by Perin et al. (9) and Gentil et al. (10).

Several methodologies have been used to assess the antimicrobial potential of different active constituents. Among these techniques, stand out the agar-diffusion test, which assess the efficiency of growth inhibition of gram-positive and gram-negative bacteria in a semi-quantitative manner, and the bioautographic method, which outlines, by means of thin layer chromatography (TLC), the chemical profile of extract constituents and phases related to the antimicrobial activity against target microorganisms.

The purpose of this study was to assess, using the agar-diffusion method, the *A. lappa* rough extract phase that presents antimicrobial activity against microorganisms found in endodontic infections. From

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this analysis, a bioautographic assay was performed to correlate antimicrobial substances present in the elected phase and in its sub-fractions with the exhibited antimicrobial action.

MATERIAL AND METHODS

Preparation of Arctium lappa Extract. *A. lappa* leaves were obtained from the Medicinal Plants collection of the University of Ribeirão Preto (UNAERP) at Ribeirão Preto, SP, Brazil in September 2003.

The leaves were dried in a ventilation stove and macerated with ethanol/water (7:3) in an amber container for 48 h. The resulting tincture was concentrated in a rotating evaporator and then lyophilized. The phases were obtained from liquid/liquid partition in a separating funnel by adding 150 mL distilled water and then successively the following solvents: hexane, ethyl acetate and butanol. In this way, after evaporation and lyophilization, aqueous, hexanic, ethyl acetate and butanolic phases were obtained, respectively.

Agar-Diffusion Test. The agar-diffusion test was performed with the rough phases resulting from the partition of *A. lappa* extract. First, the stock samples were prepared by diluting 10 mg of each *A. lappa* phase in 1 mL of propyleneglycol. The target microorganism cultures were prepared separately in 15 mL of semi-solid *Brain Heart Infusion* (BHI) medium, adjusted by tube 1 of the MacFarland scale ($3x10^8$ cfu/mL) and the set was poured on Petri plates. The following pathogens, obtained from the *American Type Culture Collection* (ATCC), were used in this study: *Enterococcus faecalis* (ATCC 29210), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633) and *Candida albicans* (ATCC 10231).

Fifteen plates were prepared, each containing five equidistant 0.5-cm diameter wells in which 50 μL of each test sample were poured and one of them received propyleneglycol only (solvent control). Thereafter, the plates were incubated at 37ºC for 24 h.

Hexanic Phase Column Chromatography Fractioning. Because the agar-diffusion test evidenced that the hexanic phase was a growth inhibitor for most of the target microorganisms, the hexanic phase was fractioned. For this purpose was used a silica gel 60 column eluted with hexane:ethyl acetate mixtures in increasing order of polarity (Table 1).

Bioautographic Assay. The *A. lappa* hexanic phase and its sub-fractions were subjected to bioautographic assay. The samples (20 mg) were diluted in 1 mL chloroform and applied on glass plates with silica gel F_{254}. The plates were developed in mobile hexane:ethyl acetate phase (7:3) and then covered with Agar BHI containing 3×10^8 cfu/mL of each microorganism suspension separately.

This procedure was duplicated. After 24 h at 37ºC, each bioautogram was dyed by a 2,3,5-triphenyltetrazolium chloride (TTC) aqueous solution in order to observe the inhibition areas. To assess the chemical profile of the samples, a replicate of the chromatographic plate was developed simultaneously to be revealed by the vanillin/sulphuric acid coloring reagent, followed by heating.

RESULTS

The means of antimicrobial activity of the *A. lappa* phases, obtained from the agar-diffusion test, are given in Table 2.

The microbial inhibition zones exhibited by the *A. lappa* hexanic phase constituents and its sub-fractions are shown in Figure 1, and their respective retention factors (R_f) are given in Table 3.

Figure 2 shows the chromatographic plate revealed by the vanillin/sulphuric acid color reagent.

Table 1. Eluting systems used for obtaining *A. lappa* hexanic phase sub-fractions.

Eluent	Concentration (%)	Volume (mL)	Sub-fraction
Hex	100	1000	1
Hex:AcOEt	90:10	1000	2
Hex:AcOEt	80:20	750	3
Hex:AcOEt	70:30	750	4
Hex:AcOEt	60:40	750	5
Hex:AcOEt	50:50	500	6
AcOEt	100	900	7
MeOH	100	700	8

Hex = hexane; Hex:AcOEt = hexane:ethyl acetate; AcOEt = ethyl acetate; MeOH = methanol.

Table 2. Average diameter (mm) of inhibition halos produced by *A. lappa* phases on the target microorganisms in the agar-diffusion test.

	E. faecalis	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
Aqueous	1.0	1.0	0	0	0
Hexanic	1.4	1.2	1.2	1.4	0
Ethyl acetate	1.2	1.1	0	0	0
Butanolic	1.0	1.0	0	0	0
Propyleneglycol	0	0	0	0	0

Table 3. Retention factors (R_f) of the *A. lappa* hexanic phase and its sub-fractions against the target microorganisms.

Microorganisms	Hexanic phase	1	2p	2	3	4	5	7	8
E. faecalis	0.11	0.11	0.11	0.11	0.11	0.11	0.11	-	0.11
	0.25								
	0.39			0.39	0.39	0.39	0.39		
P. aeruginosa	0.11	0	-	0.11	0.39	0.39	0.11	-	0.11
	0.25								
	0.39			0.39		0.39			
B. subtilis	0.11	0	0.04	0.11	0.11	0.11	0.11	0.04	0
S. aureus	0.11	-	-	0.11	0.11	0.11	0.11	-	-
	0.39			0.39	0.39	0.39	0.39		
C. albicans	0.11	0.11	0.11	0.11	0.11	0.11	0.11	-	0.11
	0.25								
	0.39			0.39	0.39	0.39	0.39		

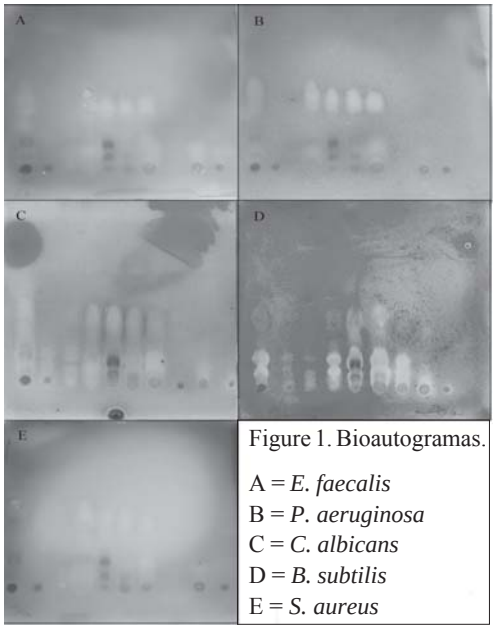

Figure 1. Bioautogramas.
A = *E. faecalis*
B = *P. aeruginosa*
C = *C. albicans*
D = *B. subtilis*
E = *S. aureus*

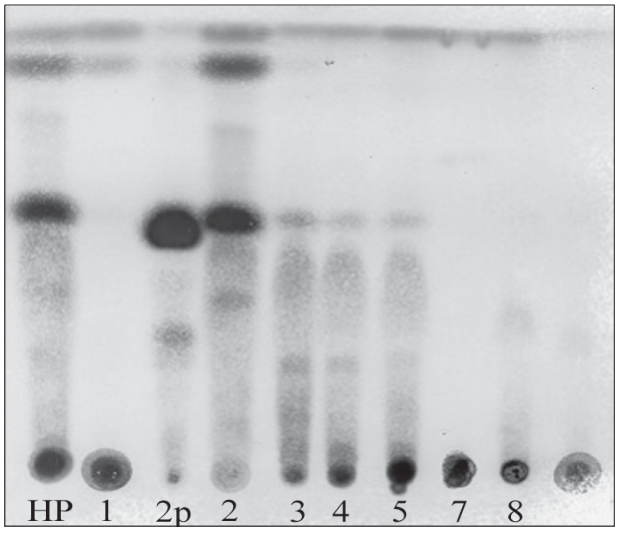

Figure 2. Chromatic profile of the hexanic phase and its sub-fractions. Developer: vanillin/sulphuric acid reagent. HP = hexanic phase; 1 to 8 = sub-fractions.

DISCUSSION

It has been demonstrated that *A. lappa* exhibits antimicrobial activity against oral microorganisms. Perin et al. (9), while evaluating three forms of the rough extract of this plant (20% tincture, extract concentrated by rotaevaporation and lyophilized extract), reported that the lyophilized extract was the most effective against *B. subtilis* and *C. albicans*. Gentil et al. (10) observed that the *A. lappa* ethyl acetate fraction used as intracanal medication for 5 days in teeth infected with *C. albicans*, *E. coli*, *L. acidophylus*, *P. aeruginosa* and *S. mutans* inhibited microbial growth after 14 days. The findings of these studies highlight the need for better knowing the chemical profile of this plant and identifying the substances that most inhibit the pathogens involved in endodontic infections.

The agar-diffusion test performed in this study showed, in a semi-quantitative manner, that the hexanic phase exhibited the greatest antimicrobial activity. Therefore, it was selected for fractioning, chemical profile analysis and bioautographic assay. This greater antimicrobial activity may be explained by the fact that the hexanic phase is extracted in the first place and presents higher concentrations of the substances that are also in the ethyl acetate phase.

Among other environmental factors, one aspect to be observed is the season when the leaves were collected. According to Freitas et al. (2004), secondary metabolite production occurs as a function of the plant-environment interaction in response to biologic and chemical factors. This fact may explain the divergent results observed from extracts of the same species but collected in different seasons.

TLC bioautographic assay allowed outlining the chemical profile of the hexanic phase constituents and its sub-fractions, thus detecting the active substances that presented antimicrobial activity. The results of this assay revealed that the analyzed samples presented different inhibition zones (Fig. 1) with distinct R_f, suggesting thereby the presence of antimicrobial active substances with different polarities (Table 3).

Additionally, the assay showed that the hexanic phase has three inhibition areas specifically. R_f 0.11 composites inhibited the growth of all tested microorganisms, R_f 0.39 composites inhibited all pathogens but *B. subtilis*, while R_f 0.25 composites inhibited *E. faecalis*, *P. aeruginosa* and *C. albicans*. The sub-fractions also presented different microbial inhibition zones, but they were not better than those obtained with the hexanic phase for the whole set of microorganisms, even though there were some specific results (*B. subtilis*, sub-fractions 1 and 8, R_f 0; sub-fractions 2p and 7, R_f 0.04).

The microbial inhibition potential of *Arctium lappa* observed in this study opens perspectives for its use as an intracanal medication.

RESUMO

A atividade antimicrobiana de extratos brutos de folhas de *Arctium lappa*, bem como de suas fases, foi avaliada in vitro. Os microrganismos *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* e *Candida albicans*, comuns na cavidade bucal, especificamente em infecções endodônticas, foram utilizados. O método de difusão em Agar permitiu a detecção da fase hexânica como inibitória do crescimento microbiano. Ensaios de bioautografia identificaram substâncias antimicrobianas presentes no extrato. Os resultados demonstraram a presença, na fase hexânica bruta e em suas sub-frações, de constituintes que têm Rf (fatores de retenção) em três zonas distintas, sugerindo a presença de ativos com estruturas químicas de diferentes polaridades, que exibiram especificidade contra os microrganismos alvos. Conclui-se que os constituintes de *Arctium lappa* apresentam um grande potencial de inibição microbiana contra os microrganismos endodônticos estudados.

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