AgroLife Scientific Journal - Volume 4, Number 2, 2015
ISSN 2285-5718; ISSN CD-ROM 2285-5726; ISSN ONLINE 2286-0126; ISSN-L 2285-5718

IN VITRO EVALUATION OF *Eupatorium cannabium* ANTIMICROBIAL ACTIVITY

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Abstract

This study aimed to prove the antimicrobial potential of different dried leaves Eupatorium cannabium extracts made of a Romanian cultivar, respectively chlorofomic extract, water extract and hydro-alcoholic extract. In the tests have taken into accoun Gram-positive test bacteria (Bacillus cereus, Staphylococcus aureus and Enterococcus faecalis), Gram-negative test bacteria (Escherichia coli) and fungus (Candida albicans and Aspergillus niger). In vitro, the antimicrobial activity was assessed by the "drop agar diffusion" method.
In the case of the chloroformic extract and hydro-alcoholic extracts of the Eupatorium cannabium local variety the inhibitory activity have been noticed only in the case of Escherichia coli and Bacillus cereus, as well as on the dimorphic yeast Candida albicans. No clear inhibition have been noticed in the case of Staphylococcus aureus, Enterococcus faecalis and Aspergillus niger.

Key words*: in vitro, Eupatorium cannabium, antibacterial, antifungal.*

INTRODUCTION

Phytotherapy, as form of traditional and conventional human and veterinary medicine, is one of the oldest and the most widely spread systems of therapy. The skill of treatment using medicinal plants was developed by all nations directly linked to the available plants. Phytotherapy is very intensively used in prophylactic purposes and with the aim of treatment of milder forms of diseases, chronic diseases and recurrent infections as well as in organic livestock production (Bojor, 2003; Davidovic, 2012).
Aromatic and medicinal plants are known to produce certain bioactive molecules which can inhibit the growth of different microoganisms (Cowan, 1999). Many extracts from medicine plant have been known to possess antimicrobial effects and used for the purpose of food preservation and medicinal purposes. Considered as time tested and comparatively safe both for human used and for environment the herbal extracts have received much attention as a source of new antibacterial drugs (Arvind, 2011).

The literature reported *Eupatorium* sp. as a source of materials as flavonoids (e.g., quercetin, hypersoide, jaceosidin, kaempferol), ternary compounds (e.g., taraxasterol palmitate, b-taraxasterol), sesquiterpenes (e.g., eeupalinolide A and eupalinolide B) and volatile oil.

Figure 1. Aerial part of *Eupatorium canabium*

These active ingredients produce varied pharmacological effects such as disinfection, antiinflammation, anti-neoplasm, immunoregulation, liver and damage protection, blood glucose decrease (Kazuo,

Yoshihisa, & Mitsumasa, 1979; Xu et al., 1998; Yan et al., 2003). Moreover, extracts of *Eupatorium lindleyanum* are proposed to be used as food additive (Li et al., 2008), while essential oil of *Eupatorium canabium* can be employed during food storage against *Aspegillus* development and aflatoxin formation (Kumar et al., 2007).

Eupatorium canabium, commonly known as Hemp-agrimony, is a robust perennial plant of *Asteraceae* family (Figure 1). Is native in many areas of Europe and is traditionally know for its use in tumor inhibition, hypertensions and diabetes; its toxicity comes from the presence of tumorigenic pyrrolizidine alkaloids. Sometimes the leaves are used as a substitute for tea.

The presence of antimicrobial and other biological activities have been already demonstrated in different extracts of *Eupatorium* spp. Gupta et al. (2002) have noticed that petroleum ether extract of *Eupatorium ayapana* showed higher antibacterial and antifungal activity than the methanolic extract. In 2008, Ji et al. have reported the antimicrobial activity of water decoct of *Eupatorium lindleyanum* DC on tested Gram-positive bacteria (*S. aureus, Bacillus subtilis, Bacillus cereus* and *Enterococcus faeciumand*) and Gram-negative species (*Escherichia coli, Salmonella typhimurium, Proteus vulgaris* and *Pseudomonas fluorescens*). Meanwhile, Arvid and Amit (2011) have proved the antibacterial (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella aerogenes* and *Pseudomonas* aeruginosa) and antifungal (*Aspergilus niger, Aspergilus candidus* and *Candida albicans*) effect of petroleum ether extract from *Eupatorium adenophorum* Spreng. Also, animal-studies and in vitro experiments with plant extracts of *Eupatorium perfoliatum* both indicate antiinflammatory effects beside antiplasmodial effect against *Plasmodium falciparum* (Hensel et al., 2011). Also, anitbacterial activity have been proven on resinous exudate from twigs and leaves of *Eupatorium salvia* (Urzua et al., 1998).

Up to know few studies have been reported on the antimicobial activities of *Eupatorium cannabium.*

In this study we have focused on extracts of local Romanian variety of *Eupatorium cannabium* which have been tested for the anitmicrobial activity on Gram-positive and Gram-negative bacteria, as well as on yeast and moulds.

MATERIALS AND METHODS

Collection of plant material

Plants of a local Romanian variety of *Eupatorium cannabium* have been harvested from the experimental field of Dacia Plant S.A., Bod, Brasov County (Centre-Romania) during the month of the plants have been cultivated under certified ecological conditions. Herbarium specimen was preserved at the manufacturer.

Preparation of plant extracts

The dried leaves of *Eupatorium cannabium* have been grounded into powder. Three type of extracts have been prepared by our partners (ICCF Bucharest) as follows: E1 was chloroformic extract E 62.5 mg/mL PPG 50%; E2 was water extract E 250 mg/mL PPG 20%; E3 was hydro-alcoholic extract E 250 mg/mL PPG 20%.

Test microorganisms

In the test have been taken into account different type of microorganisms (Table 1), respectively three Gram-positive test bacteria (*Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecalis*), one Gram-negative test bacteria (*Escherichia coli)* and two fungus (*Candida albicans* and *Aspergillus niger*).

The bacterial strains were maintained and tested on nutrient agar (0.5% peptone; 0.3% yeast extract; 1.5% agar; 0.5% NaCl) at 37^{o}C, using 24 hours old inoculums.

Yeasts and fungal strains were maintained and tested on Yeast Extract Glucose Agar (1% yeast extract; 2% peptone; 2% glucose; 2% agar) at 30^{o}C, using 24 hours old inoculums for yeasts or mycelium block (5 mm) for filamentous fungi.

Table 1. Microorganisms tested for their susceptibility to *Eupatorium canabium* alcoholic extracts

No.	Strain	Characteristics	Origin
1.	*Bacillus cereus* CP1	*Gram*-positive, beta hemolytic bacterium	Collection of Faculty of Biotechnology, Bucharest, Romania
2.	*Staphylococcus aureus* CP 4	*Gram*-positive, catalase-positive	Collection of Faculty of Biotechnology, Bucharest, Romania
3.	*Enterococcus faecalis* CP 3	*Gram*-positive, sensible to β-lactam-based antibiotics	Collection of Faculty of Biotechnology, Bucharest, Romania
4.	*Escherichia coli* CP2	*Gram*-negative, toxinogenic serotype	Collection of Faculty of Biotechnology, Bucharest, Romania
5.	*Candida albicans* ATCC10231	Serotype A, sensible to nystatin	Collection of Microbial Genetics and Biotechnology, Faculty of Biology, Bucharest, Romania
6.	*Aspergillus niger* F2T	Responsible for pulmonary infections in dogs	Collection CBM Biotehgen Bucharest, Romania

Antimicrobial Analysis

In vitro, the antimicrobial activity was assessed by the "drop agar diffusion" method (Figure 2). The microorganisms were spread using 100 µl of suspension containing 10^8 CFU/ml on nutrient agar or on Yeast Extract Glucose Agar respectively. Concerning *A. niger* F2T, the mycelium block (5 mm) was placed in the centre of the medium in Petri dishes.

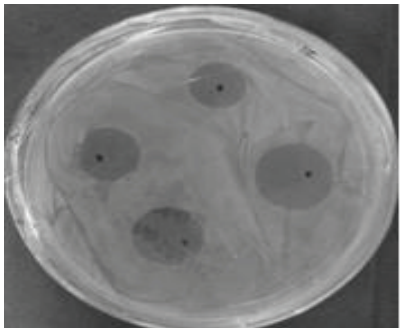

Figure 2. Aspects from the "drop agar diffusion" screening method

After 1 hour of incubation at 35°C for bacteria and 28°C for fungi, spots of 10 µL and 20 µL of each sterile extract have been added in the agar plates pre- inoculated with the tested microorganisms.

Non diluted ampicillin (for bacteria) and fluconazole (for fungi) have been used as positive control and the *Eupatorium* extracts solvent have been used in the master plates.

The diameter of growth inhibition zones (halos) was measured in millimetres on two axes and the mean value reported. The results are the mean of two separate experiments with three repetitions for each sample.

RESULTS AND DISCUSSIONS

In this study three different extracts of *Eupatorium cannabium* (chloroformic, water extract and hydro-alcoholic extract) have been tested for their antimicrobial activity on different type of microorganisms, respectively three Gram-positive test bacteria (*Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecalis*), one Gram-negative test bacteria (*Escherichia coli*) and two fungus (*Candida albicans* and *Aspergillus niger*).

Antimicrobial activity of *Eupatorium cannabium* extracts on pathogenic bacteria

The *Eupatorium cannabium* extracts have been tested on Gram-positive test bacteria (*Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecalis*) and one Gram-negative test bacteria (*Escherichia coli*).

The results are summarized in Table 2. In the case of the Gram-negative bacteria (*Escherichia coli* CP2) all the extracts has shown inhibitory activity in both applied concentrations (Figure 3).

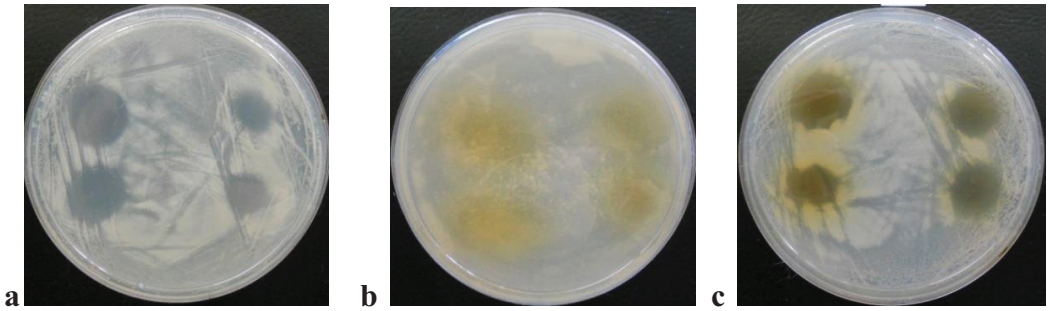

Figure 3. Inhibitory activity of *Eupatorium cannabinum* extracts on test strain of *E. coli*
(a - E1 chloroformic extract, b - E2 water extract, c - E3 hydro-alcoholic extract)

On the Gram-positive bacteria side, the most inhibited was the strain test of *Bacillus cereus* CP1, all the extracts having low to moderate inhibitory activity; the most efficient has been the hydro-alcoholic extract (Figure 4). Higher concentrations should have been tested further.

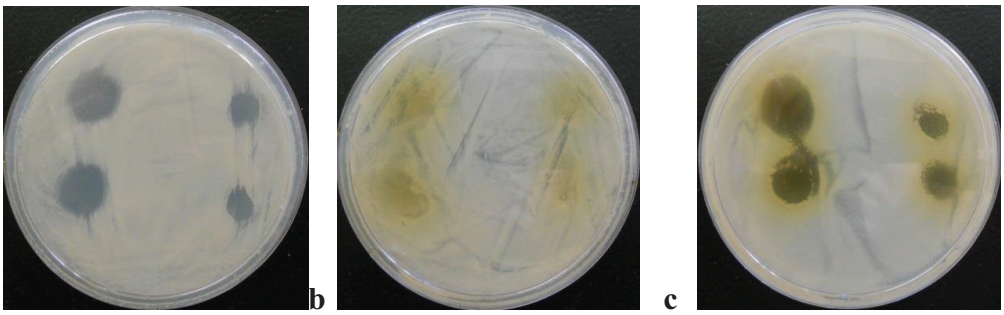

Figure 4. Inhibitory activity of *Eupatorium cannabinum* extracts on test strain of *Bacillus cereus*
(a - E1 chloroformic extract, b - E2 water extract, c - E3 hydro-alcoholic extract)

Table 2. Antibacterial activity of *Eupatorium cannabinum*

Tested bacteria	*Eupatorium cannabinum* extracts					
	E1 (chloroformic extract)		E2 (water extract)		E3 (hydro-alcoholic extract)	
	10 µl	20 µl	10 µl	20 µl	10 µl	20 µl
E. coli CP2	+	+++	+	++	+	+++
B. cereus CP 1	+	++	+	+	+	++
S. aureus CP 4	-	-	-	-	+	+
E. faecalis CP 3	-	-	-	-	-	+

Legend: - = no halo formation (no inhibition);
+ = low inhibitory activity;
++ = moderate inhibitory activity;
+++ = high inhibitory activity

Regarding the other two tested Gram-positive bacteria, *Staphyloccocus aureus* and *Enterococcus faecalis*, low inhibition has been noticed in the case of double concentrated hydro-alcoholic extracts.

Some authors have reported by inhibitory activity of *Eupatorium* sp. extracts on pathogenic bacteria, as water decoct of *Eupatorium lindleyanum* (Ji et al., 2008) or as petroleum ether extract of *Eupatorium adenophorum* Spreng (Arvid and Amit, 2011). Antibacterial screening of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of *Eupatorium glandulosum* leaves exhibited a broad spectrum of inhibitory activity against Gram (+) and Gram (-) pathogenic bacteria in a study conucted by Sasikumar et al. (2005).

While in the reported results *Eupatorium* extracts have inhibited the growth of all the pathogenic bacteria we have taken in account in our study (*Escherichia coli, Bacillus cereus, S. aureus, Enterococcus* sp.), in the case of the chloroformic extract and hydro-alcoholic extracts of the local variety of *Eupatorium cannabium* the inhibitory activity have been noticed only in the case of *Escherichia coli* and *Bacillus cereus*. This results can be linked to the solvent and the extraction method, but also can be a matter of the resistance of the tested strains.

Antimicrobial activity of *Eupatorium cannabium* extracts on pathogenic fungi

The *Eupatorium cannabium* extracts have been tested on on one dimorphic yeast, *Candida albicans* and a micotoxinogenic strain of *Aspergillus niger* (Table 3).

As shown in Figure 5, the dimorphic yeast *Candida albicans* has been inhibited by the chloroformic and hydro-alcoholic extracts, while the water extracts has no influence on the microorganism growth.

Table 3. Antifungal activity of *Eupatorium cannabinum*

Tested fungi	*Eupatorium cannabinum* extracts					
	E1 (chloroformic extract)		E2 (water extract)		E3 (hydro-alcoholic extract)	
	10 µl	20 µl	10 µl	20 µl	10 µl	20 µl
Candida albicans ATCC10231	++	+++	+	+	+	++
Aspergillus niger F2T	-	-	-	-	-	-

Legend: - = no halo formation (no inhibition);
+ = low inhibitory activity;
++ = moderate inhibitory activity;
+++ = high inhibitory activity

Concerning the philamentous fungus, *Aspergillus niger*, no inhibitory activity has been noticed in all the extracts cases. Our results are only partially in accordance with results obtained by Arvid and Amit (2011) which have proved antifungal activity of petroleum ether extract from *Eupatorium adenophorum* Spreng on *Aspergilus niger* and *Candida albicans*.

This emphasize the fact that the plant origin and the extraction method are important factors when preparing an antimicrobial product.

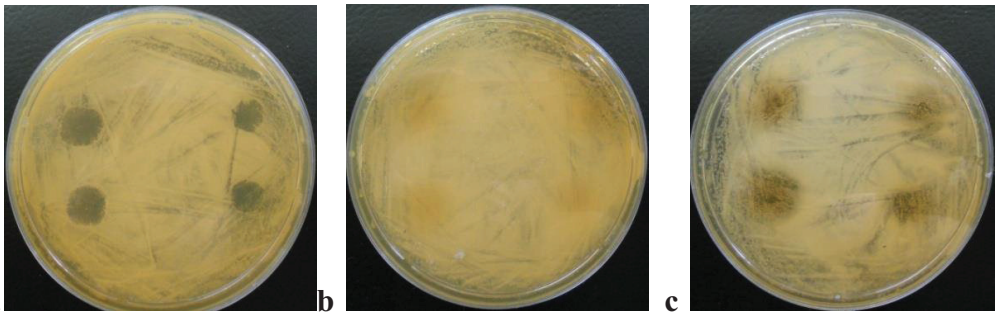

Figure 5. Inhibitory activity of *Eupatorium cannabinum* extracts on test strain of *Candida albicans*
(a - E1 chloroformic extract, b - E2 water extract, c - E3 hydro-alcoholic extract)

CONCLUSIONS

This study aimed to prove the antimicrobial potential of different dried leaves *Eupatorium cannabium* extracts made of a Romanian cultivar, respectively chlorofomic extract, water extract and hydro-alcoholic extract.

In the tests have taken into accoun Gram-positive test bacteria (*Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecalis*), Gram-negative test bacteria (*Escherichia coli)* and fungus (*Candida albicans* and *Aspergillus niger*).

While former reported results showed antibacterial activity of water decoct of *Eupatorium lindleyanum* (Ji et al., 2008) and petroleum ether extract from *Eupatorium adenophorum* Spreng (Arvid and Amit, 2011) on a wider range of Gram-negative and Gram-positive bacteria, in the case of the chloroformic extract and hydro-alcoholic extracts of the *Eupatorium cannabium* local variety the inhibitory activity have been noticed only in the case of *Escherichia coli* and *Bacillus cereus*. This results can be linked to the solvent and the extraction method, but also can be a matter of the resistance of the tested strains.

Regarding the antifungal activity, our results are partially in accordance with results obtained

by Arvid and Amit (2011) which have proved antifungal activity of petroleum ether extract from *Eupatorium adenophorum* Spreng on both *Aspergilus niger* and *Candida albicans*, while in the case of the tested chloroformic extract and hydro-alcoholic extracts of the *Eupatorium cannabium* the inhibitory activity has been noticed only in the case of *Candida albicans*. This emphasize the fact that the plant origin and the extraction method are important factors when preparing an antimicrobial product.

Further investigation have to be performed in different extraction conditions and solvent and the test of MCI should be applied in order to be able to recommend the leaves of *Eupatorium cannabium* extracts in the animal or human phytotherapy.

ACKNOWLEDGEMENTS

This work was supported by the Executive Agency for Higher Education, Research, Development and Innovation Funding in the frame of Partnerships in S&T priority domains Program, Project no. 134/2012, acronym PHYTOIMMUVET.

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