P69: ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF ITALIAN UNIFLORAL HONEYS EXTRACTS AGAINST DIFFERENT PATHOGENIC SPECIES

Sateriale D.[1)], Scioscia E.[1),#)], Colicchio R.[2)], Pagliuca C.[2)], Paolucci M.[1)], Varricchio E.[1)], Volpe M.G.[3)], Salvatore P.[2),4)], Pagliarulo C.[1),)]*

[1)] University of Sannio, Department of Science and Technology – Via Port'Arsa, 11, 82100, Benevento (Italy)
[2)] Federico II University, Department of Molecular Medicine and Medical Biotechnologies – Via S. Pansini, 5, *80145,* Napoli (Italy)
[3)] Institute of Food Science-CNR – Via Roma, 64, 83100, Avellino (Italy)
[4)] CEINGE - Advanced Biotechnologies s.c.ar.l. – *Via G. Salvatore, 486, 80145,* Napoli (Italy)
[#)] Present address: Cremonini S.P.A. – Via Modena, 53, 41014, Castelvetro di Modena, Modena (Italy)
[*)] Corresponding author – E-mail address: caterina.pagliarulo@unisannio.it (Caterina Pagliarulo, MD, PhD)

Abstract – The phenomenon of antibiotic resistance, that appears increasingly alarming worldwide, has renewed the interest in the study of many natural extracts with antimicrobial properties, such as honey. Even if several honeys have been approved for clinical using, there are some obstacles for therapeutic applicability of honey in medicine, and not only. The variability of antimicrobial activity of different honeys and the incomplete knowledge of the involved antimicrobial compounds are the major ones.

The aim of this study has been to define the antimicrobial profile of hydro-alcoholic extracts prepared from Italian unifloral honeys, particularly acacia, chestnut and sulla. The antimicrobial capability of hydro-alcoholic extracts was evaluated against clinical isolates of different bacterial species involved in human pathology, such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*. In addition, the antifungal effect of honeys extracts was verified against the UEF88662-*Aphanomyces astaci* and SMM2-*Fusarium avenaceum* strains, both fungal species responsible of different diseases in crayfish and plants. *In vitro* preliminary microbiological results demonstrated that unifloral honeys hydro-alcoholic extracts from Campania are able to effectively counteract the growth and survival of different pathogenic microrganisms.

Keywords: natural extracts, unifloral honeys, antimicrobial activity, sporicidal effect

1. INTRODUCTION

Since long time, honey is greatly used for its nutritional value and for its therapeutic properties. Recent scientific research has revalued this natural product showing that the non-heated natural honey has a significant antibacterial activity [1]. Instead, on antifungal activity of honey, the current investigation is still not very thorough.

Although the use of honey for medical purposes has a long history, resistant microbial species to it have not been identified yet [2]. The absence of microbial resistance to honey, together with its high biocompatibility, make this natural product a promising alternative to the conventionally used antimicrobial agents [3], both for exclusive using and in combination with them.

Numerous differences were found between the composition and the properties of the many varieties of existing honey, which reflect the characteristics of the respective sources of botanical origin [4]. Among the many types of honeys produced in the Italian territory the acacia honey (*Robinia pseudoacacia L.*), the chestnut honey (*Castanea sativa*) and the sulla honey (*Hedysarum coronarium*) are of particular importance, especially due to the high concentration of polyphenols [5]. In this context, the characterization of the antimicrobial properties of various types of natural honey is of particular relevance.

2. EXPERIMENTAL

Three different honey samples were used to perform microbiological tests, in particular acacia, chestnut and sulla, all produced from a company in the south of Italy, named 'Company Vignogna Angelo', located in the High Tammaro area, in Benevento (Italy). The hydro-alcoholic honey extracts were prepared in the Chemistry Laboratory of the Institute of Food Science (ISA) of the CNR in Avellino. The hydro-alcoholic buffer used for the

extraction was produced from 50% ethanol/water (v/v).

The antimicrobial effects of hydro-alcoholic extracts of unifloral honeys has been evaluated against several pathogen bacteria and fungi. Particularly, to assess the antibacterial activity, the clinical isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*, kindly provided by the U.O.C. of Clinical Microbiology of A.O.U Federico II of Naples (Italy), were tested. To assess the antifungal activity, the fungal strains UEF88662 of *Aphanomyces astaci* and SMM2 of *Fusarium avenaceum*, provided by the Department of Animal Biology Kuopio Campus of the University of Eastern Finland, were used. The microorganisms were cultured in broth and agar media at 20-37°C. The Columbia CNA agar (OXOID), Trypticase Soy agar (OXOID) and Brain Heart Infusion agar/broth (CONDA) media were used for bacterial strains, while Potato Dextrose (PD) agar/broth (CONDA) medium was used for fungal strains.

To qualitatively evaluate the antimicrobial effects exerted by the hydro-alcoholic honey extracts against selected microorganisms, an antimicrobial activity *in vitro* assay was performed using the agar diffusion method, similar to that described by Kirby-Bauer [6].

The quantitative evaluation of antimicrobial effects of the extracts was carried out by growth and survival assays. Bacterial and fungal sensitivity to different extracts concentrations were determined using the tube diluition method with standard inoculum $1*10^5$ UFC/mL (Units forming colonies/mL) according to the international guidelines issued by the CLSI (Clinical and Laboratory Standards Institute) [7] [8].

Ampicillin and tioconazole were used as positive control for bacterial and fungal isolates, respectively. The hydro-alcoholic buffer of extraction was used as negative control.

The inhibitory effect of the acacia honey extracts on fitness of the two fungal strains was performed monitoring the fungal growth and survival in the presence of increasing concentrations of extracts, during the observation period of 96 h.

After evaluating the inhibitory effects of the acacia honey extracts on the vegetative forms of the two fungal isolates, the ability of the extracts to inhibit the production of fungal spores was evaluated by a sporulation inhibition assay. To induce spores production, a variation of the procedure described by Makkonen et al. [9] was followed. For the sporulation inhibition assays, the acacia honey extracts were added at different concentrations. The fungal spores count was performed under optical microscope (Nikon Eclipse E600) with a Burker chamber.All experiments were performed in triplicate, with three independent cultures. The results obtained were analyzed and graphically reported by using ''GraphPad Prism 6'' software; the statistical significance was determined by the Two-way ANOVA test with a Bonferroni correction (p-value ≤ 0.05).

3. RESULTS AND DISCUSSION

The preliminary data showed that hydro-alcoholic honey extracts of acacia, chestnut and sulla exhibited an antimicrobial activity both on bacterial and fungal isolates. Antimicrobial activity of each extract was expressed as the diameter of the inhibition zones measured around each paper disc containing the natural extract, as shown in Table 1. The honey extracts effectively counteract both the growth of Gram-positive and Gram-negative bacteria. *Staphylococcus aureus* and *Staphylococcus epidermidis* have been shown to be particularly sensitive. In addition, also the growth of the two fungal species was inhibited by the extracts, and *Fusarium avenaceum* was particularly sensitive.

Table 1 Antimicrobial activity of unifloral honey extracts determined with the agar diffusion method*

Microbial strain	AHE (20 mg/disc)	CHE (20 mg/disc)	SHE (20 mg/disc)	AMP (100 µg/disc)	TCZ (1.4 mg/disc)
S. aureus	13.00 ± 4.24	22.50 ± 3.54	16.50 ± 2.12	45.00 ± 7.07	-
S. epidermidis	23.00 ± 4.24	18.00 ± 11.31	23.00 ± 4.24	20.00 ± 4.24	-
E. coli	22.50 ± 3.54	15.50 ± 0.71	20.00 ± 0.00	33.00 ± 3.83	-
A. astaci UEF88662	20.00 ± 7.07	17.50 ± 3.54	20.00 ± 0.00	-	20.00 ± 7.07
F. avenaceum SMM2	20.00 ± 7.07	20.00 ± 0.00	22.50 ± 3.54	-	40.00 ± 7.07

* The inhibition zone (mm) is reported as mean of triplicate assay ± standard deviation. AHE, Acacia Honey Extracts; CHE, Chestnut Honey Extracts; SHE, Sulla Honey Extracts; AMP, Ampicillin; TCZ, Tioconazole.

Table 2 Quantitative evaluation of antimicrobial activity of unifloral honey extracts

Microbial strain	AHE		CHE		SHE		AMP		TCZ	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC	MIC	MFC
S. aureus	15	30	15	25	15	25	0.01	0.02	-	-
S. epidermidis	15	25	15	50	15	25	0.32	3.2	-	-
E. coli	15	30	15	30	15	30	0.01	0.08	-	-
A. astaci UEF88662	10	20	10	20	10	20	-	-	1.5	15
F. avenaceum SMM2	10	20	10	20	10	20	-	-	1	2

AHE, Acacia Honey Extracts; CHE, Chestnut Honey Extracts; SHE, Sulla Honey Extracts; AMP, Ampicillin; TCZ, Tioconazole. MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; MFC, Minimum Fungicidal Concentration. MIC, MBC and MFC are reported as μg/μL.

The values of minimum inhibitory concentrations (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) obtained by testing the hydro-alcoholic honey extracts and the control substances against the microbial isolates are shown in Table 2. The quantitative assay confirms the preliminary tests. The MIC, MBC and MFC of the hydro-alcoholic extracts of the unifloral honeys showed low values with broad-spectrum of bacteriostatic and bactericidal activity, in particular against both staphylococci species. In addition the same extracts showed also a good fungicidal and fungistatic activity.

These results are according with the recent scientific research. A lot of laboratory studies and clinical trials have shown that honey is an effective antibacterial agent. Several natural honeys, such as the *Leptospermum scoparium (L. scoparium*) honey have been reported to have an inhibitory effect on around 60 species of bacteria, including aerobes and anaerobes, gram-positives and gram-negatives, due to hydrogen peroxide and the so-called 'non-peroxide inhibins' [10].

The inhibitory effect of acacia honey extracts on growth and survival of strain UEF88662 of *Aphanomyces astaci* (Figure 1 A-B), and of strain SMM2 of *Fusarium avenaceum* (Figure 1 C-D) was shown in Figure 1. According to the previous quantitative assay, the growth of fungal cells was already been inhibited at a concentration of 10μg/μL of extracts. The fitness of the two fungal isolates is compromised in a dose dependent manner when in combination with the extracts. These results indicate that the acacia honey extracts inhibit the growth and survival of *A. astaci* and *F. avenaceum* with fungistatic and fungicidal effect during the 96 h of the observation.

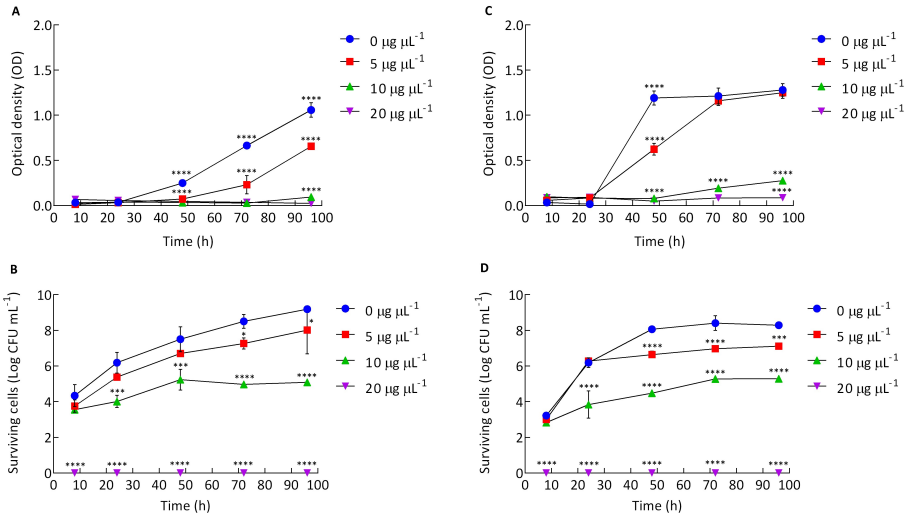

Figure 1 Inhibitory effect of acacia honey hydro-alcoholic extracts on growth (A) and survival (B) of strain UEF886622 of *Aphanomyces astaci* and on growth (C) and survival (D) of strain SMM2 of *Fusarium avenaceum*. Asterisks indicate statistical significance (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001).

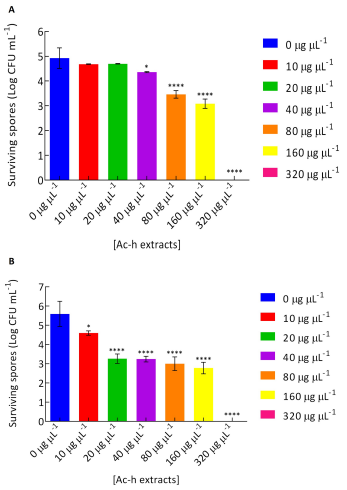

Figure 2 Inhibitory effect of acacia honey hydro-alcoholic extracts on *in vitro* sporulation of strain UEF88662 of *Aphanomyces astaci* (A) and of strain SMM2 of *Fusarium avenaceum* (B). Asterisks indicate statistical significance (*, P < 0.05; ****, P < 0.0001).

The results of the sporulation inhibition assay showed that the extracts inhibit in a dose-dependent manner both the sporulation of *A. astaci* that of *F. avenaceum* (Figure 2). The complete inhibition of the spores production is obtained with a extracts concentration of 320 µg/µL for both fungi. The antifungal effect is a remarkable and unexpected result. Since sporulation is crucial to the spread of these pathogens, particularly interesting is the sporicidal properties of these extracts.

4. CONCLUSIONS

These findings have confirmed the already known antibacterial activity of honey against human pathogens. But especially, it highlighted for the first time the remarkable antifungal activity of hydro-alcoholic honey extracts of acacia, chestnut and sulla from Campania, against two highly infectious and dangerous pathogenic fungal species, *Aphanomyces astaci* and *Fusarium avenaceum*. These pathogens are responsible for serious pathologies of invertebrates, which cause an high mortality rate, mainly in crayfish aquaculture, with strict repercussions both from the ecological point of view and economically. These results encourage to promote the development of natural principals obtained from honey for treatment and prophylaxis of different diseases, both in the medical and in the zootechnical fields, in alternative to the traditionally chemical agents, often effective but very toxic for living beings and for the environment.

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REFERENCES

[1] Lusby P.E., Coombes A.L., Wilkinson J.M. (2005). Bactericidal activity of different honeys against pathogenic bacteria. Arch. Med. Res., 36: 464-467.
[2] Cooper R.A., Jenkins L., Henriques A.F., Duggan R. S., Burton N.F. (2010). Absence of bacterial resistance to medical-grade Manuka honey. Eur. J. Clin. Microbiol. Infect. Dis., 29: 1237-1241.
[3] Bogdanov S., Jurendic T., Sieber R., Gallmann P. (2008). Honey for nutrition and health: a review. J. Am. Coll. Nutr., 27: 677-689.
[4] Molan P.C., Cooper R.A. (2000). Honey and sugar as a dressing for wounds and ulcers. Trop. Doct., 30,: 249-250.
[5] Colucci G., Varricchio E., Paolucci M., Coccia E., Viola C., Russo F., Polese M.C., Volpe M.G. Traceability makers in Italian unifloral honeys. 2nd IMEKOFOODS Promoting Objective and Measurable Food Quality & Safety. October, 2nd-5th 2016, Benevento (Italy).
[6] Bauer A.W., Kirby W.M.M., Sherris J.C., Turck M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 36: 493-96.
[7] Varaldo P.E. (2002). Antimicrobial resistance and susceptibility testing: an evergreen topic. Journal of antimicrobial chemotherapy, 50: 1-4.
[8] CLSI (Clinical and Laboratory Standards Institute) (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard-Ninth Edition, M07-A9, Vol. 32 No. 2.
[9] Makkonen J., Jussila J., Koistinen L., Paaver T., Hurt M., Kokko H. (2013). Fusarium avenaceum causes burn spot disease syndrome in noble crayfish (Astacus astacus). Journal of Invertebrate Pathology, 113: 184-190.
[10] Tan H.T., Rahman R.A., Gan S.H., Halim A.S., Hassan, S.A., Sulaiman S.A., et al. (2009). The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. BMC Complement. Alternat. Med., 9: 34.