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EVALUATION OF PROPOLIS FOR ANTIBACTERIAL ACTIVITY *IN VITRO*

EVALUAREA *IN VITRO* A ACŢIUNII ANTIBACTERIENE A PROPOLISULUI

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ABSTRACT

The antimicrobial action of the propolis tincture was tested on six bacterial strains, of which three strains were standard and three strains were isolated by the researchers. The collection strains were: *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615 and *Salmonella typhimurium* AT CC 14028. The isolated strains tested during the investigations from the Clinic of Infectious Diseases were strains of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Esherichia coli.* Raw propolis tincture produced the inhibition of the growth of bacterial cultures on the Müller-Hinton agar for four of the bacterial strains studied. For two of the bacterial strains studied, propolis tincture produced no inhibition of the growth of bacterial cultures. In the case of the bacterial strains *S. aureus* ATCC 25923 and *S. aureus*, the inhibition of growth occurred up to a dilution of 1/32, and for the bacterial strains of *S. pyogenes* ATCC 19615 and *S. typhimurium* ATCC 14028, the inhibition occurred only up to a dilution of 1/2. For the bacterial strains of *Pseudomonas spp.* and *Escherichia coli*, the inhibition of bacterial cultures did not occur. Within the *Pseudomonas spp.* strain only slight inhibition of pigmentogenesis was observed. The investigations performed in this study demonstrated that the propolis tincture *in vitro* has antibacterial effect.

Given the results from this study, the propolis tincture can be recommended and used as a local treatment, for various bacterial diseases in animals.

Keywords: propolis tincture, antibacterial activity, treatment, animal diseases

REZUMAT

Acțiunea antimcrobiană a tincturii de propolis a fost testată asupra a 6 tulpini bacteriene, dintre care trei tulpini standard și trei tulpini izolate de noi. Tulpinile de colecție au fost: *Staphylococcus aureus* ATCC 25923*, Streptococcus pyogenes* ATCC 19615*, Salmonella typhimurium* ATCC 14028, iar tulpinile izolate în cadrul Clinicii de Boli infecțioase au fost tulpini de: *Staphylococcus aureus, Pseudomonas aeruginosa* și *Escherichia coli*. La 4 din tulpinile bacteriene luate în studiu tinctura de propolis brută a produs inhibarea creșterii culturilor bacteriene pe agarul Müller-Hinton. La două din tulpinile bacteriene luate în studiu tinctura de propolis nu a produs inhibarea creșterii culturilor bacteriene. În cazul tulpinilor bacteriene *S. aureus AT CC 25923* și *S. aureus* inhibarea creșterii a avut loc până la diluția de 1/32, iar în cazul tulpinilor bacteriene *S. pyogenes ATCC 19615* și *S. typhimurium ATCC 14028* inhibarea s-a produs doar până la diluția 1/2. În cazul tulpinilor bacteriene *Pseudomonas spp.* și *Escherichia coli* nu s-a produs inhibarea creșterii culturilor bacteriene. În cadrul tulpinii de *Pseudomonas spp.* s-a putut observa doar ușoara inhibare a pigmentogenezei. Investigațiile efectuate dovedesc faptul că *in vitro* tinctura de propolis are efect antibacterian.

În concluzie, având în vedere rezultatele obținute, tinctura de propolis poate fi recomandată și folosită în tratarea locală a diferitelor afecțiuni cu etiologie bacteriană la animale.

Cuvinte cheie: tinctură de propolis, acțiune antibacteriană, tratament, boli ale animalelor

In the specialized literature, among the many reports that have been published so far, the majority have been concerned with the presence and the development of the antimicrobial resistance to antibiotics. Furthermore, it should be emphasized that the impact of antibiotic resistance on chemotherapy has been studied (5, 9, 10). It is no surprise that the antibiotic resistance has mainly occurred due to the excessive use of antimicrobial agents (11). As a consequence, there is a high risk of rapid spread of the disease caused by bacteria and a difficulty to combat the bacterial diseases (6). In order to counteract the phenomenon of antibiotic resistance, alternatives have recently been sought.

The aim of this study was to evaluate, using laboratory methods, the antimicrobial effect of the propolis tincture, by taking into consideration its beneficial therapeutic effects (1, 2, 3, 4, 11).

To achieve this goal, the sensitivity of isolated bacterial strains from animal disease cases to the action of

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the propolis tincture was tested. Additionally, the sensitivity of some standard bacterial strains to the action of the propolis tincture was also under scrutiny.

MATERIALS AND METHODS

Testing the sensitivity of the bacterial strains to the propolis tincture

In this study the antibacterial action of the propolis tincture has been tested. Additionally, the antibacterial action of the alcohol used in the preparation of the propolis tincture has been evaluated.

Obtaining the propolis tincture

The propolis tincture was obtained by mixing 220 g of raw propolis with 600 ml of ethyl alcohol of 96º v/v, allowing the mixture to macerate for eight days. After that period of time, the liquid was separated from the sediment. During the maceration period, the sealed glass container was shaken at least three times each day.

The propolis tincture container was stored in a dark, dry place at room temperature (1, 2, 8).

The propolis tincture obtained after eight days was divided into 30 ml vials.

Microbial culture

To achieve the proposed objectives of this study, six bacterial strains were studied. The collection strains were: *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615 and *Salmonella typhimurium* ATCC 14028. The isolated strains tested during the investigation, from the Clinic of Infectious Disease, were *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.*

Preparation of microbial cultures required for the disk-diffusometry antibiogram

After obtaining the pure bacterial broth culture from each tube, a certain amount of colonies were taken and dissolved in 5 ml of sterile saline until a turbidity of 0,5 on the McFarland scale was achieved, corresponding to a bacterial concentration of 10^8 UFC/ml. From this suspension, 50 µl was transferred into a 10 ml simple nutrient broth. The final inoculum concentration was 10^5 UFC/ml.

The inoculum had to be used within 15 minutes of obtaining it. The inoculum was poured into Petri dishes, which contained 4 mm thick Muller-Hinton agar, and dried. For *Streptococcus pyogenes*, ATCC 19615 the Muller-Hinton agar was used with 5% calf defibrinated blood.

Wells of 5 mm were made into agar approximately 3 cm apart. In the wells, 50 µl of the dilutions of the propolis tincture and the undiluted propolis tincture and alcohol were placed. Attention was given so that the drop did not exceed the surface of the agar.

Preparation of the propolis tincture dilutions

Dilutions of the propolis tincture were made in plastic plates with wells (plates for ELISA technique). The dispersion of materials in the plates was:

In well A, 200 µl undiluted propolis tincture was placed;

In well D1, 200 µl of alcohol was placed;

In wells A2-A6 and D2-D6, 100 µl Müller-Hinton broth were placed. In these wells serial dilutions were made, taking 100 µl of the substance from the first well and transferring 100 µl onto the next well.

After obtaining the dilutions from each well, 50 µl of diluted propolis tincture, 50 µl of undiluted propolis tincture, and 50 µl of alcohol were placed separately into wells within the Petri dishes with Müller-Hinton agar inoculated with bacterial strains.

The prepared Petri dishes were placed in a thermostat and incubated for 24 hours at 37°C. After incubation, the plates were examined by direct observation of the plate and by examination through a stereoscopic magnifying glass.

Disk-diffusometry technique for testing the sensitivity of the bacterial strains

The disk-diffusometry technique was made from subcultures, according to the requirements of the Clinical and Laboratory Standards Institute.

This type of antibiogram is performed on solid media and is based on the property of substances used to diffuse into the culture medium that is inoculated with the bacterial strain (that will be tested).

In this study, known quantities of substances with potential antibacterial effect were used (7).

The culture medium used to carry out the present investigation was the Müller-Hinton agar, with 5% calf defibrinated blood, and the simple Müller-Hinton agar (considered the best medium for routine sensitivity tests) since it ensures good reproductibility and provides good conditions for the growth of bacterial strains (7, 10, 12).

Reading the result of the antibiogram consisted in assessing the size of the inhibition zone induced by the investigated substances, an area lacking microbial colonies. The diameter of the inhibition zone is directly proportional with the sensitivity of the germen.

The result was rated by measuring the diameter of the inhibition zone (expressed in millimeters) which

subsequently was encoded in terms of susceptible, moderately susceptible, and resistant (7).

RESULTS AND DISCUSSIONS

Following the investigations, the results were systematized, processed, and presented in the form of tables and graphs. In Table 1 and Fig. 1, the results obtained are presented in order to determine the effect of inhibiting the growth of bacterial strains caused by the action of the propolis tincture.

By analysing the data, it was concluded that the bacterial strains tested reacted differently to the propolis tincture as follows:

➢ The development of *Staphylococcus aureus* ATCC 25923 was inhibited by the propolis tincture at all dilutions: the diameter of the inhibition zone was 15 mm for the raw propolis tincture and dilution of 1/2; the diameter of the inhibition area was 12 mm at dilution 1/4. For dilutions of 1/8, 1/16 and 1/32, the diameters of the inhibition zone were 11 mm, 9 mm and 7 mm, respectively.

➢ The wild strain of *Staphylococcus aureus* isolated from diseased animals showed identical behavior compared with the standardized strain of *Staphylococcus aureus*.

➢ The growth of the strain *Streptococcus pyogenes* ATCC 19615 was inhibited only by the raw propolis tincture and 1/2 dilution. For the raw propolis tincture, the diameter of the inhibition zone was 11 mm and at 1/2 dilution, 9 mm. The growth of the bacterial strains was not inhibited for the rest of the dilutions used in this study.

➢ The growth of the strain *Salmonella typhimurium* ATCC 14028 was inhibited by the raw propolis tincture and 1/2 dilution. Both inhibition areas have a diameter of 7 mm.

➢ The growth of *Pseudomonas spp.* and *Escherichia coli* has not been affected by the presence of the propolis tincture. The pigmentogenesis for *Pseudomonas spp.* was inhibited only around the area of inhibition of the raw propolis tincture and a 1/2 dilution.

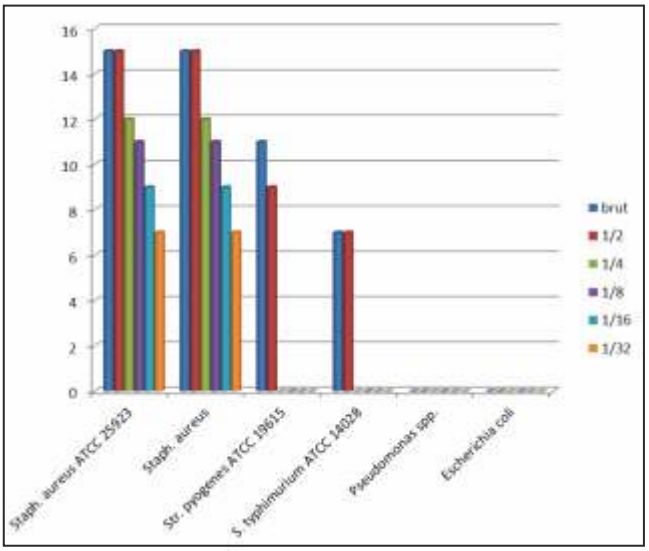

Fig. 1. Behaviour of bacterial strains relative to various dilutions of the propolis tincture

In order to distinguish between the antibacterial effect of propolis from the propolis tincture and alcohol from the propolis tincture, the 96º v/v alcohol was used in the preparation of the propolis tincture.

Diluted alcohol, as well as propolis tincture, was deposited in wells within the Petri plates previously inoculated with the microbial strains.

The 96º v/v alcohol used in the preparation of the propolis tincture has no inhibitory effect on the growth of the standard strain of *Staphylococcus aureus* ATCC 25923. Furthermore, it was determined in this study that crude (undiluted) alcohol has a very weak inhibitory effect on the strains of *Staph. aureus, Pseudomonas spp.*, *S.typhimurium* ATCC 14028. The crude (undiluted) alcohol has no effect on the *E. coli* strain as well.

The results obtained in this investigation on the antibacterial effect of alcohol are due to the fact that alcohol evaporates in a relatively short time period from Petri dishes with bacterial cultures incubated at 37°C. Alcohol, once evaporated, will not prevent the growth of bacterial cultures, which explains the existence of bacterial colonies around the wells where alcohol was deposited. Knowing the disinfectant effect of alcohol, we'd expected a strong inhibitory effect on the growth of bacterial cultures.

Table 1

Diameter of inhibition zone (in mm) obtained for the propolis tincture

Propolis tincture /dilutions	Raw	1/2	1/4	1/8	1/16	1/32
Staph. aureus ATCC 25923	15	15	12	11	9	7
Staph. aureus	15	15	12	11	9	7
Str. pyogenes ATCC 19615	11	9	0	0	0	0
S. typhimurium ATCC 14028	7	7	0	0	0	0
Pseudomonas spp.	0	0	0	0	0	0
Escherichia coli	0	0	0	0	0	0

We concluded that the same phenomenon of alcohol evaporation was present in the case of the propolis tincture, but after evaporation of alcohol in the wells, the propolis remained.

Therefore, the experimental model used in the present study quantifies the antibacterial effect of propolis from the propolis tincture.

Regarding the antimicrobial action of propolis, similar results have been obtained by other authors. Rahman M et al. have shown that propolis has a high antibacterial activity on *Staph. aureus* at a concentration level of 2.74-5.48 mg/ml, but they have obtained weak results with respect to the *E. coli* strain (9).

Rindt et al. demonstrated that propolis at 1/8 dilution has a bactericidal effect on a very resistant strain of *Staph. aureus*, isolated from a case of dermatitis in dogs (10).

The action of the propolis tincture on gram-positive bacterial strains is greater than on gram-negative bacterial strains (3, 12).

Given the inhibitory effects of the propolis tincture on the development of bacterial cultures *in vitro*, we consider that these effects should be maintained *in vivo,* at least for situations that allow for the external use of the propolis tincture.

In fact, we consider that this explains the local therapeutic success of the propolis tincture (11).

CONCLUSIONS

Research on the *in vitro* antibacterial effects of the bees propolis (for six bacterial strains) has shown that, on four bacterial strains studied, the raw propolis tincture produced inhibition of the growth of bacterial cultures around the wells that contained propolis.

For two of the bacterial strains investigated, the propolis tincture did not inhibit the growth of bacterial culture around the wells that contained propolis.

For the bacterial strains of *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus*, the inhibition of growth occurred up to a dilution of 1/32.

In case of the bacterial strains of *Streptococcus pyogenes* ATCC 19615 and *Salmonella typhimurium ATCC* 14028, inhibition occurred only up to dilution 1/2.

In the case of the bacterial strains of *Pseudomonas spp.* and *Escherichia coli*, inhibition of the growth of bacterial cultures did not occur.

Within the *Pseudomonas spp* strain, only slight inhibition of pigmentogenesis was observed.

The investigations performed in this study demonstrate that *in vitro* the propolis tincture has an antibacterial effect.

Taking into account the results obtained, the propolis tincture can be recommended and used in the local treatment of various animal bacterial diseases.

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