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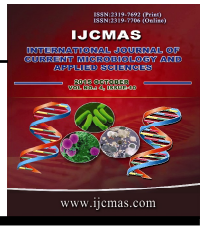

Original Research Article

Antimicrobial Effect *in vitro* of Aqueous Extracts of Leaves and Branches of Willow (*Salix babylonica* L.)

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A B S T R A C T

Studies were carried out to determine the *in vitro* antimicrobial activity of dried twigs with leaves of weeping willow *Salix babylonica L.* collected in the spring and summer in order to assess the possibilities to obtain antimicrobials, as well as of its epizootic safety when is used as an energy crop production of biogas. Used were 20 pathogenic bacterial strains belonging to the species *Escherichia coli, Salmonella enterica, Staphylococcus aureus, Paenibacillus alvei* and *Candida albicans.* Experiments were carried out by the classical agar-gel diffusion method of Bauer-Kirby and by the method for determining the Minimum Inhibitory Concentrations (MICs). It was found that the twigs and leaves of the plant exhibit antimicrobial activity *in vitro* with respect to bacteria and oval fungi if these are harvested in the summer (at the middle of June). The inhibition zones at application of the aqueous extract thereof were with an average diameter of 13.38±2.22 mm. The effect was more pronounced against Gram-negative bacteria (*E. coli* and *S. enterica*) and was similar to this of the broad spectrum antibiotic thiamphenicol. High sensitivity manifested and *C. albicans,* and lowest - *P. alvei* (10.75±1.29 mm). The average value of the MIC_{50} of the extract of the material obtained in the summer was 70.4±17.41 mg/ml and that is an indicator of good antimicrobial activity *in vitro.* This action was less pronounced in the water extracts of twigs and leaves of *S. babylonica,* collected in the spring (March and April).

Introduction

Weeping willow *Salix babylonica L.* is widespread in Bulgaria tree species preferring wetlands. It is a dioecious plant whose flowers are gathered in catkins and appear in early spring together with the leaves. Their smell is pleasant, have a sweet juice and sooner attract bees and other insects. Pollination is mainly done by the wind. The seeds are small and ripen in May-June. Willow is a hydrophilic and grows along rivers, streams and in swampy areas, but also occurs in the mountains up to 1600 m altitude. It develops quickly and lives up to 100 years. Its wood is soft, rots easily, but is used for the production of furniture, in light constructions and others. From young

willow branches is obtained the substance salicyl, from which is produced the medicament aspirin (Tonkov *et al.,* 2005).

Extracts of some plants such as *S. babylonica* are applied as a food additive for ruminants, as help increase the intake of dry matter from the feed and the milk production. Rivero *et al.* (2012) found that an extract of *S. babylonica*, used as an additive to improve the digestibility of the feed, does not show negative impact on blood parameters and on the health of lambs.

There is evidence that extracts of weeping willow can exhibit antiparasitic action. According to research of Hernandez *et al.* (2014) the number of protozoa in the rumen of lambs decreased significantly after two months of oral administration of extract from the leaves of *S. babylonica* in an amount of 30 ml per day. Sati *et al.* (2013) reported a good antimycotic activity of 20% ethanolic extract of *S. babylonica* against *Fusarium oxysporum*, unlike such extract of *Triumfetta pillosa*. However, no reports yet about studies of weeping willow for antibacterial properties. Because extracts of some plants exhibit such biological effects (Popova and Michailov, 2000; Michailov *et al.,* 2001; Popova and Baykov, 2013; Popova *et al.,* 2013; Saadoun *et al.,* 2014), the purpose of this work was to perform studies of aqueous extracts of weeping willow for antimicrobial activity *in vitro* against antibiotic-resistant pathogenic microorganisms, which are a serious global problem in modern medical and veterinary medical practice. The increasing prevalence of resistance to most antimicrobial means requires search of new effective compounds with antibacterial effect that bypass the constructed microbial resistance mechanisms. Moreover, today with the increase of trust in non-traditional medicine as an alternative form of health care, the researches of medicinal plants for active ingredients are increased.

That is why this work is directed towards study of aqueous extracts of twigs with leaves of *Salix babylonica* for possible antimicrobial effect on pathogenic bacteria and fungi. To assess the antimicrobial effect, the presence of data for the minimum inhibitory concentrations for microorganisms from different groups is essential. Since such data are not found in the available literature, the purpose of these studies is to determine the smallest concentrations that prevent the multiplication of Gram-positive and Gram-negative microorganisms *in vitro*. These studies are promising both in terms of enrichment of the pharmacognosy of medicinal plants, and to implement the mixed substrates containing willow (such as silage, etc.) at anaerobic digestion of organic waste (manures and sewage sludge).

Materials and Methods

Plant extracts

The effect of aqueous extracts of dried twigs with leaves of weeping willow *Salix babylonica* L., collected in spring and summer, was tested. A comparative study of the materials obtained at different periods of development with an interval of about 3 months was made: No. 1 - at the middle of March with flower catkins (at the beginning of the period of leafing and flowering); No. 2 – at the end of April and No. 3 – at the middle of June with seeds (during the ripening seeds). The materials were cut and dried, and from them were prepared 20% extracts by boiling for 5 min in distilled water, followed by filtration through a sterile gauze and sterilization by Koch. The resulting aqueous extracts had a pH of 6.5.

Control

The antibiotic thiamphenicol was used as a control in determining the antimicrobial effect of the substances because of its broad spectrum of activity and since most of used strains showed sensitivity to it *in vitro*.

Microorganisms

In the studies were used 20 strains of various Gram-negative and Gram-positive microorganisms, respectively by 4 strains of the species *Escherichia coli, Salmonella enterica, Staphylococcus aureus, Paenibacillus alvei* and *Candida albicans.* They were isolated from animals with various chronic infections (*P. alvei* – from honeycombs with European foulbrood) and showed multi-resistance to antibiotics of various groups *in vitro*.

Antimicrobial tests

The classical *agar-gel diffusion* method of Bauer *et al.* (1966) was used. Microbial suspensions were inoculated at a dose of 2.10^6 cells/ml on Mueller-Hinton's agar (Antisel - Sharlau Chemie S. A., Spain) with pH 7.2 – 7.4 in Petri dishes with diameter 9 cm. The extracts and control were applied by dropping by 0.1 ml (30 μg of antibiotic) in 9-mm wells in the agar with a thin agar layer at the bottom. Results were recorded by measuring the diameters of inhibitory zones in mm, including the hole diameter, after incubation during 24–48 h at 37^oC. According to the tree-degree system of Bauer-Kirby, inhibitory effect was established at zones > 12 mm for the extracts and >17mm for the antibiotic.

The determination of the *minimum inhibitory concentrations* (MICs) was performed by the method of twofold serial dilutions on Mueller-Hinton's agar (Antisel - Sharlau Chemie S. A., Spain) with pH 7,2-7,4, as per Ericsson and Sherris (1971) and NCCLS M7-A. Bacterial suspensions were applied at a dose 10^6 cells/ml. After incubation at 35–37°C for 18 to 24 hours the number of developed colonies was determined. The extracts were inflicted in double increasing concentrations from 64 to 2048 μg/ml agar. MIC_{50} were calculated mathematically depending on the number of the colonies of the agar medium with the respective extract or antibiotic dilution, compared to the control medium colonies without drugs. Absence of antimicrobial activity of the extracts was read at MIC>100 mg/ml, and antimicrobial effect – at MIC≤100 mg/ml, which was defined as high at MIC≤50 mg/ml.

Statistical analysis

The results were analyzed statistically by the classical method of Student-Fisher.

Results and Discussion

In the research conducted by the diffusion method of Bauer *et al.* (1966) was found that some of the extracts exhibited antimicrobial effect. The results are presented in table 1.

From the generalized data is shown that the most pronounced antimicrobial activity manifested extract No. 3, derived from material collected in June, and and weakest – No. 1, received in March. The differences between them, however, were not significant (P>0.5). The results show high sensitivity to the extracts of the Gram-negative bacteria and *C. albicans* and lower - of the tested staphylococci and bacilli. Inhibition zones of extract No.3 for *E. coli* even outperformed these of the control antibiotic, which is indicative of a very good antibacterial effect. The strains of Gram-positive bacteria *S.*

aureus and *P. alvei*, used in these studies, did not show sensitivity to the tested aqueous extracts. However, they were not influenced significantly and by the action of the control antibiotic owing to their multi resistance. Interestingly, *Candida albicans* showed almost equally high sensitivity to the effect of the three tested extracts.

The results of the tests to determine the minimum inhibitory concentrations of the aqueous extracts of the leaves of weeping willow, detected for 20 strains from 5 different species of Gram-positive and Gram-negative pathogens, are shown in table 2.

The results confirm those obtained by the agar-gel diffusion method. As seen from the aggregated data, well expressed inhibitory action showed the extract of the material collected during the month of June, and the weakest effect - that of the earliest collected leaves immediately after unfurling the willow in March. The differences were significant - between MICs of extracts No. 1 and No. 3 $P<0.001$, and between No. 2 and No. 3 - $P<0.01$. The antimicrobial effect of the extract No. 3 was best expressed against Gram-negative bacteria. MICs for them had the lowest values. Most strongly affected were the strains of *E. coli* and of the oval fungus *C. albicans*, and the least - the tested bacilli of the species *P. alvei*. Although MICs of extract No. 3 were higher than those of the antibiotic thiamphenicol, the differences were not statistically significant ($P>0.5$). The rest two extracts had significantly higher MICs than those of the antibiotic ($P <0.001$).

The results of the present studies, obtained by both used classical methods, show that aqueous extracts of dried twigs with leaves of weeping willow *Salix babylonica* L., collected during the summer, exhibit antimicrobial activity. Although it is highly exhibited against Gram-negative bacteria, the spectrum of their effect is wide. It also covers Gram-positive species such as *S. aureus*, as well as oval fungi (*C. albicans*). Although the studied strains were inhibited *in vitro* by lower minimum concentrations of thiamphenicol, the fact that $MICs_{50}$ of the extracts for all of them were less than 100 mg/ml, is indicative of a good antimicrobial effect of the weeping willow. Especially promising is the established sensitivity of *E. coli* to the extracts from this plant, because the high multi resistance of its strains is a significant medical problem. Only the strains of *P. alvei* were influenced slightly by the action of these extracts. The bacilli of this kind, however, exhibit a high sensitivity to other plants such as leaves of the tree species *Paulownia elongata*, applied as an aqueous extract of the leaves (Popova and Baykov, 2013). These bacilli also exhibit *in vitro* susceptibility to the effects of other herbal preparations such as “Ecophil-P” (Popova, 2011), which is important for limiting their proliferation in the environment in presence of the relevant plants or preparations.

In the other used microorganisms our results of the research of extract No. 3 are similar to those obtained by Popova and Baykov (2013) when testing the antimicrobial activity of an aqueous extract from leaves of *P. elongata*. These authors found high sensitivity of the Gram-negative bacteria and *C. albicans* to this plant extract, while the Gram-positive staphylococci and streptococci had been proved less sensitive to it. Saadoun *et al.* (2014) reported a sensitivity of Gram-positive bacteria such as *S. aureus*, *Streptococcus* sp. and *Bacillus* sp. to alcohol extract of leaves of the exotic species *Prosopis juliflora* and to a lesser extent of Gram-negative species such as *Klebsiella* sp. and *E. coli*. Obviously, using

the alcohol is achieved a better extraction of the active components of the plants, since this way are derived not only water-soluble, but other ingredients, and while achieving a higher concentration of active substances in the extract. Other authors such as Popova and Michailov (2000) and Michailov *et al.* (2001) also reported for a significant antimicrobial effect *in vitro* of herbal preparations containing alcohol extract of various plants. They found a high antimicrobial activity against various Gram-positive and Gram-negative microorganisms of concentrated plant extracts derived from selected combinations of herbs. The present studies were performed with aqueous extracts of twigs and leaves of willow tree, as this is the most accessible and commonly used method for the preparation of extracts of medicinal plants for external and internal use in practice. Furthermore, in our opinion at testing of water extracts *in vitro* for antimicrobial action certainly is eliminated the possibility of reporting better results, due to the impact of an eventual residual effect of the alcohol after its removal by the pre-incubation for evaporation.

Table.1 Inhibitory effect of 20% aqueous extracts of twigs and leaves of weeping willow *Salix babylonica* L. (No. 1- collected in March, No. 2 - in April and No. 3 - in June) on pathogenic microorganisms in the agar diffusion method

Microorganisms	Number of strains tested	Inhibition zones in mm			
		No. 1	No. 2	No. 3	Thiam-phenicol
E. coli	4	**10,5**±1,12	**12,0**±1,58	**16,63**±6,72	**16,12**±5,37
S. enterica	4	**11,5**±2,29	**12,5**±0,5	**14,25**±0,43	**21,38**±8,2
S. aureus	4	**9,25**±0,43	**10,25**±0,43	**11,0**±0,71	**13,00**±2,18
P. alvei	4	**9,25**±0,43	**9,5**±0,5	**10,75**±1,29	**12,9**±2,69
C. albicans	4	**14,0**±7,52	**14,5**±6,8	**14,25**±6,29	**18,00**±8,39
Total	20	**10,9**±1,76	**11,75**±1,76	**13,38**±2,22	**16,20**±3,29

Table.2 Minimum inhibitory concentrations (MIC) of aqueous extracts of twigs and leaves of weeping willow *Salix babylonica* L. (No. 1- collected in March, No. 2 - in April and No. 3 - in June) for pathogenic microorganisms from different groups

Microorganisms	Number of strains tested	MIC_{50} (mg/ml)			
		No. 1	No. 2	No. 3	Thiam-phenicol
E. coli	4	**256,0**±0,0	**128,0**±0,0	**56,0**±13,85	**36,0**±35,55
S. enterica	4	**256,0**±0,0	**256,0**±0,0	**62,0**±30,39	**25,5**±17,88
S. aureus	4	**256,0**±0,0	**256,0**±0,0	**60,0**±10,58	**36,0**±35,55
P. alvei	4	**256,0**±0,0	**256,0**±0,0	**104,0**±30,98	**62,0**±41,13
C. albicans	4	**160,0**±55,4	**128,0**±0,0	**70,0**±37,47	**7,5**±5,07
Total	20	**236,8**±38,4	**204**±62,7	**70,4**±17,41	**33,3**±17,74

The data from these studies give us reason to assume that antimicrobial ingredients are contained mainly in the seeds of the weeping willow as they ripen in May-June

and are located on branches together with the leaves. The blossoms obviously do not contain antibacterial ingredients since extract No. 1, derived from material collected during flowering, showed an inhibitory effect only against *C. albicans*, but not against the tested bacteria. Obviously the antimycotic action is due to other components of the plant different than the antibacterial ingredients.

The identified antibacterial and antifungal properties of the branches and leaves of weeping willow give hope that they can be used (alone or in combination with other suitable plant materials) for the preparation of antimicrobial agents with possibilities for applications in medicine. Interesting and important from a practical point of view would be also studying the components of the plant, which show this effects, as well as the mechanisms of their action. The established by us antimicrobial activity further increases the value of the leaves of the weeping willow from a medical standpoint.

In conclusions, the aqueous extracts of dried twigs with leaves of weeping willow *Salix babylonica* L. exhibit inhibitory activity *in vitro* against Gram-positive and Gram-negative microorganisms. The highest is this antimicrobial effect of the extract of the material collected in the summer, similar to that of the control broad spectrum antibiotic thiamphenicol. Inhibition zones at its application are with diameters average 13.38±2.22 mm, and the average value of the MIC_{50} is 70.4±17.41 mg/ml. This extract shows a high inhibitory activity against Gram-negative bacteria and *C. albicans*, and less - towards staphylococci and bacilli. Weakest antibacterial action shows the extract of material obtained in early spring during leafing and flowering of the plant in March, and average effect - that obtained in April. *Candida albicans* shows almost equally high sensitivity to the action of the three tested extracts.

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