



ANTIBACTERIAL ACTIVITY OF SOME WILD MEDICAL PLANTS EXTRACT TO ANTIBIOTIC RESISTANT *ESCHERICHIA COLI*

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ABSTRACT

Antibiotics are probably the most successful family of drugs so far developed for improving human health. Because of increasing resistance to antibiotics of many bacteria, plant extracts and plant compounds are of new interest as antiseptics and antimicrobial agents in medicine. In this study, we researched antimicrobial effects of extracts of some medical plants (*Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* and *Taraxacum officinale*) from Slovakia to antibiotic resistant and antibiotic sensitive bacteria isolated from milk of cows and mare, which were bred in different conditions. Microorganisms which were used in this experiment we isolated from milk from conventional breeding of cows (ten *E. coli* strains) and from ecological breeding of Lipican mare (ten *E. coli* strains) by sterile cotton swabs. For antibiotic susceptibility testing was used disc diffusion method according by EUCAST. After dried at room temperature we weighed 50 g of crushed medical plants (parts) and it were to extract in 400 ml methanol for two weeks at room temperature. For antimicrobial susceptibility testing of medical plants extract blank

discs with 6 mm diameter disc diffusion method was used. We determined that all *Escherichia coli* strains isolated from milk of conventional breeding of cows were resistant to ampicillin and chloramphenicol. We determined that all tested ampicillin and chloramphenicol resistant *E. coli* strains isolated from conventional breeding of cow showed susceptibility to all used medical plants extracts. In difference, we determined that antibiotic susceptible *E. coli* strains isolated from ecological breeding of Lipican mare were susceptible to *Tussilago farfara* extract only. From these results we could be conclude some observations, which could be important step in treatment of bacterial infections caused by antibiotic resistant bacteria and it could be important knowledge for treatment of livestock in conventional breeding. Of course, to confirm our analysis further studies are needed.

Keywords: Antimicrobial activity, wild medical plants, *E. coli*, methanolic extracts

INTRODUCTION

Antibiotics are probably the most successful family of drugs so far developed for improving human health. Besides this fundamental application, antibiotics (antimicrobials at large) have also been used for preventing and treating animals and plants infections (McManus et al., 2002; Smith et al., 2002; Cabello et al., 2006; Singer et al., 2003). All these applications made antibiotics to be released in large amounts in natural ecosystems (Sarmah et al., 2006). These antibiotics than lead to increasing antibiotic resistance in bacteria by prolong selective pressure of antibiotics (Kolář et al., 2000). Infections caused by resistant strains of microorganisms causing costly treatment of animals and humans. Such infections prolong the pathological condition and if not treated with the right antibiotics may be increased mortality (Witte, 2006). Because of increasing resistance to antibiotics of many bacteria, plant extracts and plant compounds are of new interest as antiseptics and antimicrobial agents in medicine (Augustin and Hoch, 2004; Blaschek et al., 2004; Norton, 2000). There has been a resurgence of interest in the development of drugs deriving from plants. These plant derived substances could be found in various parts like roots, leaves, shoots and bark of plants (Salamon and Fejer, 2011; Grušová et al., 2012). Many plants are used in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy (Noumi and

Yomi, 2001). However, scientific evidence of the antimicrobial effects of many of them has not been investigated yet.

In this study, we researched antimicrobial effects of extracts of some medical plants from Slovakia to antibiotic resistant and antibiotic sensitive bacteria isolated from milk of cows and Lipican mare, which were bred in different conditions.

MATERIAL AND METHODS

Collection of samples

In this experiment we extracted five medical plants from Slovakia: *Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* and *Taraxacum officinale*. Additional information about collecting and used parts of medical plants are described in the Table 1. Medical plants was indentified by **Kresánek and Krejča (1977)**.

Table 1 Information overview about collected medical plants

Plants / information	Family	Harvest area	Used parts
<i>Tussilago farfara</i>	<i>Asteraceae</i>	Gelnica	Flower + stem
<i>Equisetum arvense</i>	<i>Equisetaceae</i>	Partizánske	Leaf + stem
<i>Sambucus nigra</i>	<i>Asteraceae</i>	Nitra	Flower
<i>Aesculus hippocastanum</i>	<i>Aesculaceae</i>	Nitra	Flower
<i>Taraxacum officinale</i>	<i>Asteraceae</i>	Nitra	Flower

Microorganisms which were used in this experiment we isolated from milk from conventional breeding of cows (ten *Escherichia coli* strains) and from ecological breeding of Lipican mare (ten *E. coli* strains) by sterile cotton swabs (Copan Inovation, Italy) in sterile transport medium and transported to Department of microbiology laboratory of Faculty of biotechnology and food sciences in SUA in Nitra.

Isolation and identification of microorganisms

Bacterial samples were spread on the surface of agar by sterile cotton swab directly. For cultivation of bacterial strains MacConkey agar (Biomark, Pune) was used. Cultivation of *Enterobacteriaceae* genera was done at 35±2 °C during 24 hours. After the first incubation

was need recultivation to obtain pure cultures of *E. coli* in the same conditions. For purifying of *E. coli* colonies and probably identification of *E. coli* strains Chromogenic coliform agar (Oxoid, UK) was used. For obtaining the pure culture of *E. coli* four-ways streak plate method was used. Every these steps of recultivation was done in the same conditions. After the purifying of individual microorganisms we identified *E. coli* by biochemical test Enterotest 24 (Erba Lachema, Brno) and TNW Lite software (Erba Lachema, Brno). Methodical procedure for Enterotest 24 is described in manufacturer manual.

Antibiotic susceptibility testing

The pure overnight inoculums of *E. coli* strains were prepared by suspending of colonies into the physiological solution from agar plates and every suspensions were adjusted to equal a 0.5 McFarland standard. The sensitivity of all *E. coli* strains was tested against: ampicillin (AMP 10) 10 $\mu\text{g}\cdot\text{disc}^{-1}$, chloramphenicol (C 30) 30 $\mu\text{g}\cdot\text{disc}^{-1}$, imipenem (IMP 10) 10 $\mu\text{g}\cdot\text{disc}^{-1}$, tetracycline (TE 30) 30 $\mu\text{g}\cdot\text{disc}^{-1}$ (Oxoid, UK). For antibiotic susceptibility testing was used disc diffusion method according by EUCAST (European Committee on Antimicrobial Susceptibility Testing). Incubation of *E. coli* strains were done at 35 \pm 2 °C on Mueller-Hinton agar (Biomark, Pune). Interpretation of inhibition zones around the disc was according by EUCAST (Breakpoint tables for interpretation of zone diameters Version 2.0, valid from 2012-01-01). The inhibition zones were controlled with the references susceptible *E. coli* CCM 3988.

Preparing of medical plants extract

After dried at room temperature we weighed 50 g of crushed medical plants (parts) and it were to extract in 400 ml methanol for two weeks at room temperature. From created extract sediments and plants residues were filtered by Whatman no. 54 filter paper (GE Healthcare, UK). The crude extracts solution were put to the glass and methanol was evaporated by rotary vacuum evaporator (Stuart RE300DB, UK) at 40 °C at -800 mbar. The pure created extract was weighed. The yields after methanol evaporation for each medical plants extracts were: *Tussilago farfara* extract (757.6 mg), *Equisetum arvense* extract (392.5 mg), *Sambucus nigra* extract (442.4 mg), *Aesculus hippocastanum* extract (509.5 mg) and *Taraxacum officinale* extract (269.7 mg). For dissolving of medical plants extracts we used

different volume of DMSO (dimethylsulfoxide) to equal value 512 µg/ml. Dissolved extracts in DMSO were storage in a fridge at -16 °C.

Antimicrobial susceptibility testing of medical plants extract

Simultaneously with antibiotic susceptibility testing pure overnight inoculums of *E. coli* strains were prepared by suspending of colonies into the physiological solution from agar plates and every suspensions were adjusted to equal a 0.5 McFarland standard. The 100 µl bacterial solutions were spread on the surface of Mueller Hinton agar (Biomark, Pune) evenly. Petri dishes were dried in dryer machine at 60 °C. For antimicrobial susceptibility testing of medical plants extract blank discs (Oxoid, UK) with 6 mm diameter disc diffusion method was used. Every discs were impregnated by 20 µl of 512 µg/ml medical plants extracts dissolved in DMSO. Therefore of each discs contained 10.24 µg active compounds from medical plant extracts. After the impregnation discs were spread on the surface of Mueller Hinton agar evenly. Antimicrobial susceptibility testing was done at 35 ±2 °C during 18-20 hours. The inhibition zones were controlled with discs impregnated with pure DMSO. After the incubation inhibition zones were measured by according EUCAST in mm.

Statistical evaluation

For this experiment we used statistical software STATGRAPHIC and we calculated some basic statistical values like: average, standard deviation, coefficient of variation, minimum, maximum and range. Equally, we determined significant differences between study groups by One-way analysis of variance with Tukey HSD Test.

RESULTS AND DISCUSSION

In this research we focused to comparison between antimicrobial effect of crude medical plants extracts to antibiotic resistant and antibiotic susceptible *E. coli* isolated from milk of conventional breeding of cow and ecological breeding of Lipican mare. After the isolation of *Enterobacteriaceae* genera strains from MacConkey agar and their purifying on the Chromogenic coliform agar we identified followed *E. coli* strain by Enterotest 24 and TNW Lite Software, which is described into the Table 2.

Table 2 Identified *E. coli* strains and itself antibiotic resistance or susceptibility profile

Microorganisms	Isolated from	AMP 10	C 30	TE 30	IMP 10	Percentage of identification
<i>E. coli</i>	Cow milk	R	R	S	S	99.98
<i>E. coli</i>	Mare milk	S	S	S	S	100

Legend: AMP 10 – ampicillin, C 30 – chloramphenicol, TE 30 – tetracycline, IMP 10 – imipenem, R – resistant, S - sensitive

We identified *E. coli* from milk samples only. We determined that all *E. coli* strains isolated from milk of conventional breeding of cows were resistant to ampicillin and chloramphenicol. Resistance to another used antibiotics were not detected. All strains of *E. coli* isolated from mare milk from ecological breeding showed no resistance to used antibiotics. Equally, **Roopnarinet al. (2005)** examined antibiotic resistance in *E. coli* isolated from milk samples and they determined ampicillin resistant *E. coli* too. Resistance to chloramphenicol determined **Hleba et al. (2010)** who isolated strains from *Enterobacteriaceae* genera from milk products.

After the antibiotic susceptibility testing we tested antimicrobial activity of medical plants extract to pure *E. coli* strains. We measured diameter of inhibition zones around the discs, which are described in the Table 3. We determined that all tested ampicillin and chloramphenicol resistant *E. coli* strains isolated from conventional breeding of cow showed susceptibility to all used medical plants extracts. In difference, we determined that antibiotic susceptible *E. coli* strains isolated from ecological breeding of mare were susceptible to *Tussilago farfara* extract only. Others used medical plants extracts had not effects to *E. coli* strains isolated from ecological breeding of mare. Not many scientists researched antimicrobial effects of similar medical plants. However, **Kokoska et al. (2002)** determined that *Tussilago farfara* extract had antimicrobial effect to *Bacillus cereus* (15.63 mg/ml) and *Staphylococcus aureus* (62.50 mg/ml) by MIC susceptibility testing. Antimicrobial effects of *Tussilago farfara* to *E. coli* they not determined. But they determined that many others medical plants had antimicrobial effects to *E. coli*.

By basic statistic (Table 3) we calculated that the most antimicrobial activity had *Tussilago farfara* extract in the both cases. Averages of inhibition zones were 16.7 ± 3.65 mm for ampicillin and chloramphenicol resistant *E. coli* isolated from conventional breeding of cows and 8.3 ± 1.41 mm for antibiotic susceptible *E. coli* isolated from ecological breeding of mare. Conversely, the smallest antimicrobial effect had methanolic extract from *Aesculus*

hippocastanum (average of inhibition zones 9 ± 1.88 mm). Effects of others medical plants extracts to antibiotic susceptible *E. coli* isolated from ecological breeding of mare were not detected. Similarly, **Basaran and Gonuz (2004)** researched antimicrobial effect of *Tussilago farfara* extract by disc diffusion method to *E. coli* and they determined 10 mm inhibition zone. In contrast with antibiotic susceptible *E. coli* isolated from ecological breeding of mare, ampicillin and chloramphenicol resistant *E. coli* isolated from conventional breeding were susceptible to others medical plants extracts (*Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* and *Taraxacum officinale*). **Milanović et al. (2007)** researched antimicrobial effects of *Equisetum arvense* extract and they determined 12.1 ± 0.5 mm. We determined 11.5 ± 2.95 mm inhibition zone. For example **Woods-Panzaru et al. (2009)** detected no antibacterial effect of *Taraxacum officinale* to *E. coli* isolates. They determined antimicrobial effect of *Alium sativum* (15-16 mm) only. We determined that *Taraxacum officinale* extract had antimicrobial effect in 12.8 ± 3.19 mm in diameter. Researchers **Hearst et al. (2010)** examined antimicrobial effect of *Sambucus nigra* extract and they determined 7 mm inhibition zone. In our experiment we used methanolic *Sambucus nigra* extract and we determined 13.5 ± 4.06 mm inhibition zone.

Table 3 Summary statistic for inhibition zones of resistant and sensitive *E. coli* isolated from conventional breeding of cow and ecological breeding of mare to medical plants extracts

Statistical value / plants	<i>Tussilago farfara</i>	<i>Equisetum arvense</i>	<i>Sambucus nigra</i>	<i>Aesculus hippocastanum</i>	<i>Taraxacum officinale</i>
ATB resistant <i>E. coli</i> isolated from conventional breeding of cow					
Count	10	10	10	10	10
Average	16.7	11.5	13.5	9.0	12.8
SD	3.65	2.95	4.06	1.88	3.19
CV	21.87%	25.68%	30.08%	20.95%	24.92%
Min	11.0	8.0	9.0	7.0	10.0
Max	22.0	17.0	20.0	12.0	19.0
Range	11.0	9.0	11.0	5.0	9.0
ATB susceptible <i>E. coli</i> isolated from ecological breeding of mare					
Count	10	10	10	10	10
Average	8.3	ND	ND	ND	ND
SD	1.41	ND	ND	ND	ND
CV	17.08%	ND	ND	ND	ND
Min	7.0	ND	ND	ND	ND
Max	10.0	ND	ND	ND	ND
Range	3.0	ND	ND	ND	ND

Legend: ATB – antibiotics, ND – not determined

By the statistical evaluation with using One-way ANOVA we determined that significant differences were between groups because P-value was 0.0001. Also we used multiple range test (Tukey HSD test) and we determined significant differences between antimicrobial effects of medical plants extracts of *Tussilago farfara* and *Equisetum arvense*, *Tussilago farfara* and *Aesculus hippocastanum*, *Sambucus nigra* and *Aesculus hippocastanum* to *Escherichia coli* isolated from milk from conventional breeding of cow. Equally, statistical significant differences were between antimicrobial effects of *Tussilago farfara* extracts and others medical plants extracts to *E. coli* isolated from milk of mare from ecological breeding, because antimicrobial effect of others used medical plants extracts were not detected. Comparison of antimicrobial effects of medical plants extracts to *E. coli* between isolates from cow milk (conventional breeding) and isolates from mare milk (ecological breeding) showed that *E. coli* isolated from conventional breeding was more susceptible to medical plants extracts like *E. coli* isolated from ecological breeding.

CONCLUSION

From these results we could be conclude some observations. We determined that medical plants extracts were more effective to antibiotic resistant *E. coli* like to antibiotic susceptible *E. coli*. It could be important step in treatment of bacterial infections caused by antibiotic resistant bacteria. Equally we determined that *E. coli* isolated from conventional breeding was more susceptible to medical plants extracts like *E. coli* isolated form ecological breeding. Apart from antibiotic resistance of bacteria, it could be important knowledge for treatment of livestock in conventional breeding. We think that animals from ecological breeding have access to live multifloral feeds and therefore microcenosis from intestinal tracts were resistant to medical plants extracts. Of course, to confirm our analysis further studies are needed.

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REFERENCES

AUGUSTIN, M. – HOCH, Y. 2004. Phytotherapie bei Hauterkrankungen. Munchen : Urban und Fischer, 2004, p. 1–7. ISBN 978-3-437-56120-7.

- BASARAN, D. – GOMEZ, A. 2004. Antimicrobial activity of certain plants used in Turkish traditional medicine. In *Asian Journal of Plants Sciences*, vol. 3, 2004, no. 1, p. 104-107.
- BLASCHEK, W. – EBEL, S. – HACKENTHAL, E. – HOLZGRABE, U. – KELLER, K. – REICHLING, J. – SCHULZ, V. 2004. Hager ROM 2004 –Hagers Handbuch der Drogen und Arzneistoffe. Berlin Heidelberg : Springer, 2004. ISBN 978-3540214687.
- CABELLO, F. C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. In *Environmental Microbiology*, vol. 8, 2006, p. 1137–1144.
- EUCAST. 2012. European Committee on Antimicrobial Susceptibility Testing (Breakpoint tables for interpretation of MICs and zone diameters Version 2.0, valid from 2012-01-01). http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf.
- GRUĽOVÁ, D. – LABUN, P. – ŠERŠEŇ, F. – ŠALAMON, I. 2012. Seasonal Variation in DPPH Scavenging Activity of *Mentha x piperita*. In *Advances in Environmental Biology*, vol. 6, 2012, n. 4, p. 1477-1480.
- HEARST, C. – MCCOLLUM, G. – NELSON, D. – BALLARD, L. M. – MILLAR, B. CH. – GOLDSMITH, C. E. – ROONEY, P. J. – LOUGHREY, A. – MOORE, J. E. – RAO, J. R. 2010. Antibacterial activity of elder (*Sambucus nigra* L.) flower or berry against hospital pathogens. In *Journal of Medicinal Plants Research*, vol. 4, 2010, no. 17, p. 1805-1809.
- HLEBA, L. – KAČÁNIOVÁ, M. – POCHOP, J. – LEJKOVÁ, J. – ČUBOŇ, J. – KUNOVÁ, S. 2011. Antibiotic resistance of *Enterobacteriaceae* genera and *Salmonella* spp., *Salmonella enterica* ser. Typhimurium and Enteritidis isolated from milk, cheese and other dairy products from conventional farm in Slovakia. In *Journal of microbiology, biotechnology and food sciences*, vol. 1, 2011, no. 1, p. 1-20.
- KOKOSKA, L. – POLESNY, Z. – RADA, V. – NEPOVIM, A. – VANEK, T. 2002. Screening of some Siberian medicinal plants for antimicrobial activity. In *Journal of Ethnopharmacology*, vol. 82, 2002, p. 51- 53.
- KOLÁŘ, M. – URBÁNEK, K. – LÁTAL, T. 2000. Antibiotic selective pressure and development of bacterial resistance. In *International journal of antimicrobial agents*, vol. 17, 2001, p. 357–363
- KRESÁNEK, J. – KREJČA, J. 1977. Atlas liečivých rastlín a lesných plodov. 1. vyd. Martin :Osveta, 1977, 768 s.

- MCMANUS, P. S. – STOCKWELL, V. O. – SUNDIN, G. W. – JONES, A. L. 2002. Antibiotic use in plant agriculture. In *Annual Review of Phytopathology*, vol. 40, 2002, p. 443–465.
- MILOVANOVIĆ, V. – RADULOVIĆ, N. – TODOROVIĆ, Z. – STANKOVIĆ, M. – STOJANOVIĆ, G. 2007. Antioxidant, antimicrobial and genotoxicity screening of hydro-alcoholic extracts of five Serbian equisetum species. In *Plants Foods Human Nutrition*, vol. 62, 2007, p. 113-119.
- NORTON, S.A. 2000. Botanical heritage of dermatology. In AVALOS, J. - MAIBACH, H.I. (Eds.), *Dermatologic Botany*. CRC Press LCC : Boca Raton.
- NOUMI, E. – YOMI, A. 2001. Medicinal plants used for intestinal diseases in Mbalmayo Region, Central Province, Cameroon. In *Fitoterapia*, vol. 72, 2001, no. 3, p.246-54.
- ROOPNARINE, R. – AMMONS, D. – ADESIYUN, A. A. 2009. Frequency of antimicrobial resistance of *E. coli* isolates from dairy farms in Trinidad by source and presence of virulence markers. In *Veterinary Archives*, vol. 79, 2009, no. 3, p. 229-243.
- SALAMON, I. – FEJER, J. 2011. Content of heavy metals in poppy seeds (*Papaver somniferum* L.). In *Advances in Environmental Biology*, vol. 5, 2011, no. 2, p. 315-319.
- SARMAH, A. K. – MEYER, M. T. – BOXALL, A.B. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. In *Chemosphere*, vol. 65, 2006, p. 725–759.
- SINGER, R. S. – FINCH, R. – WEGENER, H. C. – BYWATER, R. – WALTERS, J. – LIPSITCH, M. 2003. Antibiotic resistance – the interplay between antibiotic use in animals and human beings. In *The Lancet Infectious Diseases*, vol. 3, 2003, p. 47–51.
- SMITH, D. L. – HARRIS, A. D. – JOHNSON, J. A. – SILBERGELD, E. K. – MORRIS JR., J. G. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. In *The Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, 2002, p. 6434–6439.
- WITTE, W. 2006. Selective pressure by antibiotics use in livestock. In *International Journal of Antimicrobial Agents*, vol. 16, 2006, p. 19-24.
- WOODS-PANZARU, S. – NELSON, D. – McCOLLUM, G. – BALLARD, L. M. – MILLAR, B. CH. – MAEDA, Y. – GOLDSMITH, C. E – ROONEY, P. J. – LOUGHREY, A. – RAO, J. R. – MOORE, J. E. 2009. An examination of antibacterial and antifungal properties of constituents described in traditional Ulster cures and remedies. In *Ulster Medical Journal*, vol. 78, no. 1, 2009, p. 13–15.