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# INVESTIGATION OF ANTIBACTERIAL ACTIVITY OF SUPERCRITICAL EXTRACTS OF PLANTS, AS WELL AS OF EXTRACTS OBTAINED BY OTHER TECHNOLOGICAL PROCESSES ON SOME BACTERIA ISOLATED FROM ANIMALS

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*The multiresistance of bacteria to antibiotics, as well as the lack of new antibiotics on the market encouraged the reasearch of antibacterial activity of non-antibiotic substances including plant extracts. During the previous decades, it has been proven that extracts of certain plants have a strong antibacterial activity, but their clinical use was limited due to the presence of organic solvents. However, plant extracts obtained by the process of supercritical fluid extraction contain no traces of solvents, and the latest researches have established that they do have antibacterial effects on some gram-positive bacteria. This comparative study included extracts of Common Mullein, Angelica and Echinacea obtained by means of supercritical fluid extraction, Soxlet extraction and ultrasound-assisted extraction. The study of their antibacterial activity was performed on some strains of Staphylococcus, Enterobacter cloacae and E. coli isolated from clinical material of human and animal origin. A referential strain of S. aureus ATCC 25923 was included in the research. In the study broth macrodilution method was applied by which the MIC values of extracts were determined. The Angelica extract obtained by ultrasound-assisted extraction had the strongest antibacterial activity, i.e. the lowest MIC value of 40 μg/mL for S. epidermidis strain. The Angelica extract obtained by supercritical fluid extraction also showed substantial antibacterial activity to all Staphylococcus strains included in this study, with the MIC values of 320 to 640 μg/mL. The extracts of Echinacea and Common Mullein obtained by supercritical fluid extraction, as well as of Echinacea extract obtained by Soxlet extraction showed no antibacterial activity since the MIC values of these extracts were 2560 μg/mL or >2560 μg/mL for all bacterial strains icluded in the study.*

*Key words: herbal extracts, MIC, bacteria*

## INTRODUCTION

It is well known that the emergence of multiresistant strains of bacteria is most commonly connected with the misuse and excessive use of antibiotics, in human and in veterinary medicine alike. Even though there are over 200 kinds of antibiotics and chemotherapeutics on the market nowadays, including 50 kinds of penicillin, 70 kinds of cephalosporine and 20 kinds od quinolone, the problem of multiresistance of bacteria to antibiotics is at its peak. Multiresistant strains are multiplied daily and they inhabit farms, hospitals, schools and the environment and cause severe, in most cases fatal infections worldwide in animals and humans. Due to the lack of new antibiotics on the market, studies of antibacterial effect of non-antibiotical substances of different origin, including herbal extracts, are more present nowadays, with the objective to treat humans and animals in cases of infections induced by multiresistant strains of bacteria (Deans and Ritchie, 1987; Conner, 1993; Dorman and Deans, 2000; Burt, 2004; Glisic *et al*., 2007). Essential oils have been used for those purposes, as well. Essential oils are mixtures of volatile components, mostly phenols and terpenes which are often the carriers of the aroma and the scent of the plant. The conventional way of obtaining essential oils is by hydrodistillation. This method's disadvantage is the isolation of oil at high temperatures due to which thermic degradation of a certain number of active components occurs (Jay and Rivers, 1984). Also, the secondary herbal metabolites of larger molecular mass cannot be isolated by means of hydrodestillation. A conventional method of isolating active components from herbs is the extraction by organic solvents (methanol, ethanol, chloroform, hexane etc.). The basic defect of this method is the obtaining of extracts that contain traces of organic solvents, thus being unsuitable for pharmaceutical and nutritional uses. In addition to the problem of refining the extracts, on an industrial scale, handling large quantities of organic solvents invariably involves problems of solvents regeneration and waste sideproducts management. Also, todays' rigorous demands concerning the allowed presence of organic substances in nutritional products and pharmaceutical remedies, additionally raise the cost of organic solvents extracting processes in phases dealing with the refinement of the extracts. Because of the mentioned conventional methods disadvantages of isolating active components from herbal material, studies have been commenced with the goal of procuring a new method by which the described shortcomings would be eliminated.

Over the last decades, a procedure of extraction by dense fluids, the so-called supercritical fluid extraction (SFE), was thoroughly investigated (Glisic *et al*., 2007; Kotzekidou *et al.,* 2008). Supercritical fluid extraction is performed by using fluids in a supercritical state at temperatures higher than their critical temperature and under a pressure higher than their critical pressure. In this condition the fluid has a high density which is close to the density of the liquid, while its ability of diffusion remains good and close to that of gases. These characteristics enable easy penetration of the fluid in the supercritical state into herbal material and the extraction of secondary herbal metabolites. The separation method itself is very simple and is usually based on reducing the

pressure which turns the fluid from supercritical into gaseous state thus completely separating it from the liquid or semiliquid extract. This is the way of getting an extract completely free from traces of solvent. The most commonly used solvent is carbon dioxide. The reason for its use is the favourable values of its critical parameters (31.8$^{o}$C, 7.38 MPa), low cost, availability and non-toxicity. As a non polar solvent, it is suitable for isolation of non polar components, and by adding small amounts of polar components (co-solvents) the extraction of polar compounds can be improved. Using carbon dioxide makes it possible to perform extraction at temperatures about 40$^{o}$C, by which is avoided degradation of thermically unstable active components. By varying pressure (or density of the supercritical fluid) the extracting "power" of the solvent in the supercritical procedure is changed. Lower pressures are suitable for isolating components of smaller molecular mass, whereas, using the high-pressure extraction, the extract contains mostly components of larger molecular mass. That way, by choosing the extracting conditions (temperature and presure), it is possible to obtain an extract with the maximum content of desired active substances. The only disadvantage of industrial application of SFE, as opposed to conventional methods, is the larger investment in equipment due to working in conditions of elevated pressures. The costs of production, however, are significantly reduced, the processs is simplier and more cost-efficient and the final product is characterized by good quality. In the recent years, studies of antimicrobial activity of extracts obtained by the process of supercritical extraction have become very popular, for it has been established that these extracts have strong antibacterial effects, mainly on gram-positive bacteria (Jay and Rivers, 1984; Kotzekidou *et al.,* 2008; Mišić *et al*., 2008). A possibility of using the abovementioned extracts as auxilliary medical remedies in treating local infections in humans and animals is, therefore, being considered particularly in cases where the infections are caused by bacteria exhibiting multiple resistance to antibiotics. A possibility of using the mentioned extracts as food additives for preventing and delaying microbiological food spoiling is also being considered. This paper shows the results of comparative studies of antibacterial activity of some herbal extracts obtained by different technological processes including the process of supercritical fluid extraction.

## MATERIALS AND METHODS

*Research material*

Extracts of Common mullein, Angelica and Echinacea obtained by processes of supercritical fluid extraction, ultrasound-ethanol assisted extraction and Soxlet ethanol-extraction were used for the purposes of antibacterial activity investigation.

Dried aerial parts of Common mullein (*Verbascum thapsus* L.) and Echinacea (*Echinacea purpurea*), as well as the dried root of Angelica (*Angelica archangelica*), cultivated in Serbia, were used for experimental studies. Prior to extractions, plant material was ground and sieved. Fraction 0.315-0.5 mm was used for experimental studies. Commercial carbon dioxide (99% purity, Techno-

gas) was used for the supercritical extraction. Absolute ethanol (96%, Kemika) was used for Soxlet and ultrasound-assisted extractions.

The investigation of the antimicrobial effects of plant extracts has been performed on *Staphylococcus aureus*, *Staphylococcus epidermidis, Staphylococcus haemolyticus, Enterobacter cloacae* and *E.coli* strains of human and animal origin. A referential strain *Stahylococcus aureus* ATCC 25923 was also used in the study.

All investigated bacterial strains had been isolated from clinical material delivered to the Department of Microbiology, Faculty of Veterinary Medicine, University of Belgrade. Columbia agar with the addition of 6% sheep blood (bioMerieux), MacConkey agar (bioMerieux) and nutrient broth (BioLab) were used for the isolation of bacteria. An automatic identification system API STAPH (bioMerieux), was used for typisation of staphylococci and BBL Crystal Enteric/ nonfermenter ID kit (Becton Dickinson) was used for typisation of gram-negative bacteria. For testing the antibacterial activity of plant extracts, cation adjusted Mueller Hinton II broth was used (CAMHB, Becton Dickinson). For the purpose of testing the effect of the extracts on gram-positive bacteria, 1,6% bromcersol purple (Merck) in a final concentration of 0.2 mL/200 mL was added to CAMHB. For the purpose of testing the effect of the extracts on gram-negative bacteria, 1% pehnol red (Merck) in a final concentration of 1 mL/200 mL was added to CAMHB. Dimethyl sulfoxide, (DMSO, Sigma Aldrich) and 1-2 propanediol (Acros Organics) were used as solvents for the herbal extracts.

*Research methods*

*Supercritical fluid extraction*

Extractions with supercritical carbon dioxide were preformed in Autoclave Engineers SCE Screening System with a 150 $cm^3$ extractor vessel as previously described (Burt, 2004). The supercritical extraction screening system is designed for small batch research runs, using $CO_2$ as the supercritical medium. Liquid $CO_2$ is supplied from a $CO_2$ cylinder by a siphon tube. The $CO_2$ is pumped into the system by the liquid metering pump until the required pressure is obtained. Back pressure regulators are used to set the system pressure (in the extractor and separator). Heaters are supplied on the extractor vessel for temperature elevation. The supercritical $CO_2$ flows through the extractor and enters the separator vessel. A flowmeter is provided to indicate the flow rate of $CO_2$ being passed through the system and the flow can be adjusted by a micrometering valve.

Supercritical extract from Common mullein was obtained at 30 MPa and 313 K. Supercritical extractions from Echinacea and Angelica were performed at 15 MPa and 313 K. A sample of 40 g of ground plant material was placed in the extractor vessel and the solvent flow rate was 0.3 kg/h in all experiments.

*Ultrasonic-assisted extraction*

An ultrasonic bath was used as an ultrasound source for the ultrasound-assisted extraction experiments. An open rectangular ultrasonic cleaner bath (Bandelin Sonorex RK 52, BANDELON electronic, 35 kHz, 60 W) with a useful volume of 1.8 L (internal dimensions: 150 mm x 140 mm x 100 mm) was used to

carry out the extractions. Samples of 5 g of ground plant material were added into 200 mL of 96% ethanol in a 250 mL flask and subjected to ultrasound-assisted extraction. The flask with sample was partially immersed into the ultrasonic bath, filled with water and sonicated for 45 minutes. Subsequently, the solution was filtred and ethanol was evaporated in a rotary vacuum evaporator.

*Soxlet extraction*

Extraction with ethanol was also performed in a Soxlet type apparatus. The extraction lasted 4 hours.

*Microbiological investigations*

Conventional microbiological methods were applied for the purposes of isolation and identification of bacterial strains included in the investigation. For antibacterial susceptibility testing, broth macrodilution method was applied for determining MIC (minimal inhibitory concentration) values and in accordance with the CLSI prescription (Clinical Laboratory Standards Institute, 2008, USA). Antimicrobial effects of plant extracts were investigated in concentrations (expressed in μg/mL): 1280; 640; 320; 160; 80; 40 and 20. The extracts were previously dissolved in DMSO or 1-2 propanediol in a concentration of 5120 μg/mL, and then double dilutions of extracts down to the lowest tested concentration were prepared. The desired innoculum density of $5x10^5$ CFU/mL was achieved by preparig the suspension of bacteria of approximately $1\text{-}2x10^8$ CFU/mL, which was the density equal to McFarland standard 0.5 (Becton Dickinson). The prepared suspension was diluted 10 times, to obtain final inoculum density of approximately $1\text{-}2 \times 10^7$ CFU/mL and 50 μL of this suspension was applied to CAMHB, after which the number of bacteria in the media was approximately $5x10^5$/mL. The media were incubated on 37°C for 18 hours. For MIC values the broth with lowest oil concentration and with no visible bacterial growth, was used.

## RESULTS

*Antibacterial activity of Angelica extracts*

The results of testing the antibacterial effect of Angelica are shown in Table 1. The Angelica extract obtained by ultrasound-assisted extraction had the strongest antibacterial effect, i.e. the lowest MIC value of 40 μg/mL for the *Staphylococcus epidermidis* strain. The resulting MIC value of the abovementioned extract of 320 μg/mL for 2 strains of *Staphylococcus* also indicates solid antibacterial effect. The MIC value of the mentioned extract for gram-negative bacteria was high, >2560 μg/mL.

Angelica extract obtained by Soxlet extraction in applied concentrations failed to show antimicrobial effects on all bacterial strains included in the investigation with obtained MIC values of 2560 μg/mL or >2560 μg/mL. Angelica extract obtained by supercritical extraction at temperature of 40°C under 150 bar pressure had solid antibacterial effect with MIC values of 320 μg/mL for all

*Staphylococcus* strains included in the study except for the *Stahylococcus epidermidis* strain for which the MIC value was 1280 μg/mL. Angelica extract obtained by supercritical extraction had MIC value of >2560 μg/mL for gram-negative bacteria (Table 1).

Table 1. Results of antibacterial activity investigation of Angelica extracts

| Number | Bacterial strains | MIC values in μg/mL | | |
|---|---|---|---|---|
| | | Ultrasound with ethanol | Soxlet with ethanol | Supercritical extraction |
| 1. | *Staphylococcus epidermidis* Skin swab, dog | ≤40 | >2560 | 320 |
| 2. | *Staphylococcus haemolyticus* Ear swab, cat | 320 | 2560 | 320 |
| 3. | *Staphylococcus aureus* Milk sample, cow with masititis | 320 | 2560 | 320 |
| 4. | *Staphylococcus aureus* ATCC 25923 | 1280 | 2560 | 640 |
| 5. | *Enterobacter cloacae* Skin swab, pig | >2560 | >2560 | >2560 |
| 6. | *E.coli* Human urine | >2560 | >2560 | >2560 |

*Antibacterial activity of extracts of Common mullein*

The results of antibacterial activity investigation on Mullin extracts are shown in Table 2. The Mullein extract obtained by ultrasound extraction showed moderately strong antibacterial effect on gram-positive bacteria with MIC values of 640 μg/mL for 3 tested strains of *Staphylococcus* including the referential strain, and 1280 μg/mL for *S.epidermidis* strain. The extract had no effect on gram-negative bacteria included in the study, according to obtained MIC values of >2560 μg/mL for all strains.

The Mullein extract obtained by Soxlet extraction also showed solid antibacterial activity with MIC value of 160 μg/mL for *Staphylococcus aureus* ATCC 25923, 320 μg/mL for all strains of *Staphylococcus haemolyticus and Staphylococcus aureus and* 640 μg/mL for *Staphylococcus epidermidis*. This extract also showed no effect on *Enterobacter cloacae* and *E.coli*, with obtained MIC value of >2560 μg/mL.

Supercritical extract of Mullein showed no antibacterial effect in the applied concentrations because the resulting MIC value of this extract was >2560 μg/mL for all investigated strains of bacteria (Table 2).

Table 2. Results of antibacterial activity investigation of Mullein extracts

| Number | Bacterial strains | MIC values in μg/mL | | |
|---|---|---|---|---|
| | | Ultrasound with ethanol | Soxlet with ethanol | Supercritical extraction |
| 1. | *Staphylococcus epidermidis* Skin swab, dog | 1280 | 640 | >2560 |
| 2. | *Staphylococcus haemolyticus* Ear swab, cat | 640 | 320 | >2560 |
| 3. | *Staphylococcus aureus* Milk sample, cow with masititis | 640 | 320 | >2560 |
| 4. | *Staphylococcus aureus* ATCC 25923 | 640 | 160 | >2560 |
| 5. | *Enterobacter cloacae* Skin swab, pig | >2560 | >2560 | >2560 |
| 6. | *E.coli* Human urine | >2560 | >2560 | >2560 |

*Antibacterial activity of Echinacea extracts*

The results of antibacterial activity investigation of Echinacea extracts are shown in Table 3. Echinacea extract obtained by ultrasound extraction showed a solid effect only on *S.epidermidis* strain with obtained MIC value of 320 μg/mL, whereas MIC values for all the other tested strains were 2560 μg/mL or >2560 μg/mL. The supercritical extract of Echinacea and the Echinacea extract obtained by Soxlet extraction in the applied concentrations failed to show antimicrobial effects on all bacterial strains included in the investigation with obtained MIC values of 2560 μg/mL or >2560 μg/mL.

Table 3. Results of antibacterial activity investigation of Echinacea extracts

| Number | Bacterial strains | MIC value in mg/μL | | |
|---|---|---|---|---|
| | | Ultrasound with ethanol | Soxlet with ethanol | Supercritical extraction |
| 1. | *Staphylococcus epidermidis* Skin swab, dog | 320 | >2560 | >2560 |
| 2. | *Staphylococcus haemolyticus* Ear swab, cat | 2560 | >2560 | 2560 |
| 3. | *Staphylococcus aureus* Milk sample, cow with masititis | >2560 | >2560 | 2560 |
| 4. | *Staphylococcus aureus* ATCC 25923 | 2560 | > 2560 | 2560 |
| 5. | *Enterobacter cloacae* Skin swab, pig | >2560 | >2560 | >2560 |
| 6. | *E.coli* Human urine | >2560 | >2560 | >2560 |

Figures 1, 2 and 3 show the comparative results of antibacterial activities of plant extracts categorized according to the technological extraction processes.

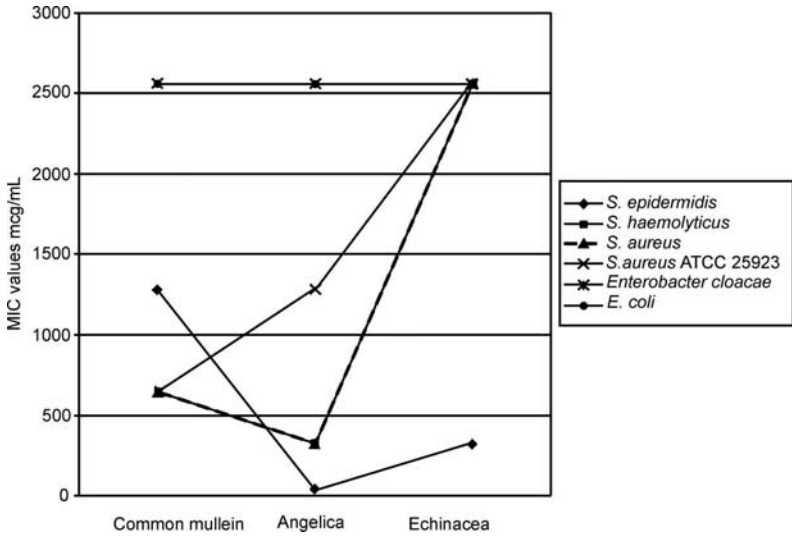


Figure 1. MIC values of extracts obtained by ultrasound-assisted extraction from Angelica, Common Mullein and Echinacea

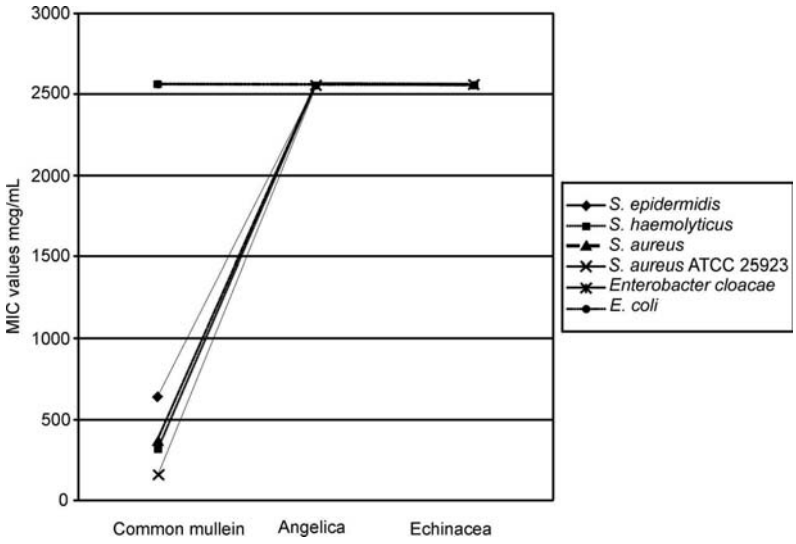


Figure 2. MIC values of extracts obtained by Soxlet extraction from Angelica, Common Mullein and Echinacea

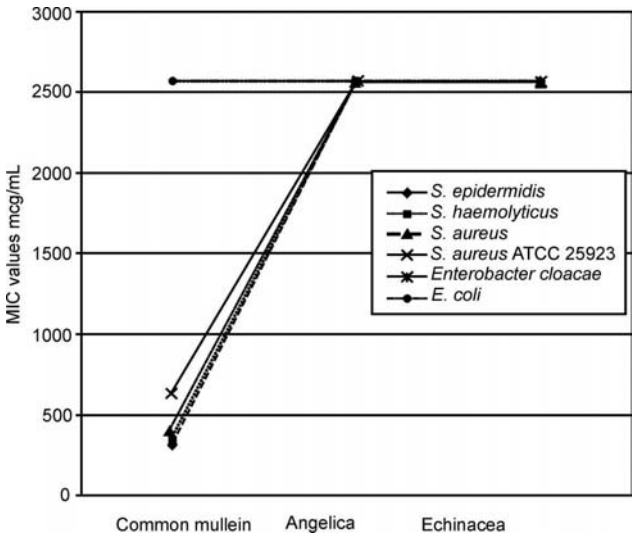


Figure 3. MIC values of extracts obtained by supercritical extraction from Angelica, Common Mullein and Echinacea

## DISCUSSION

There are no standardized methods for testing the bacterial susceptibility to plant extracts. Over the last decades, investigations of antibacterial activity of essential oils and other plant extracts were mostly improvised and the testing results were vague and confusing. The setback in these studies was the presence of organic solvents, ether, chloroform, acetone or alcohol in herbal extracts, which themselves have antibacterial effects, therefore a precise interpretation of the results was difficult. Other than that, organic solvents themselves are generally toxic to the host cells so the practical use of these extracts with strong antibacterial effect was limited, as well. In this investigation, broth microdilution method was performed according to prescribed references by CLSI for bacterial susceptibility to antibiotics investigation. A single modification of the method concerned the fact that the plant extracts were used instead of antibiotics, but the principle of the procedure, as well as the means of preparation and culture media were not altered.

As stated above, the extracts of plants obtained by supercritical extraction do not contain traces of organic solvents. They are purified substances with no additives and they do not require purity calculation (the procentage of foreign substances or active substances presence when analysing their antimicrobial effect); the product itself – the supercritical extract reffered as a pure active substance. Not all supercritical extracts are in the same aggregate state. They can be liquid or solid, depending on pressure and temperature under which the process of supercritical extraction was performed. Co-extraction of waxes and

fatty oils is one of the difficulties in testing the antimicrobial activity of supercritical extracts because they do not dissolve in water, and the methods for determination of MIC values are based on dissolving substances whose MIC value is tested in water solutions or liquid culture media. Therefore, DMSO and 1-2 propanediol, which have no antibacterial activity, were used as solvents. Certain authors recommend heating the culture media and extracts for easier dissolving, but this was not applied due to the fact that many of the extracted ingredients are thermically unstable and would disintegrate or deactivate at higher temperatures. In this study, even powdery, dry extracts were obtained, which dissolve in water completely; the extracts of Common Mullein, for instance, obtained by ultrasound-assisted extraction and Soxlet extraction, hence no solvents were used for these extracts.

By comparing the results of this study with the results of earlier reserches conducted in Serbia, it can be noticed that not all extracts obtained by supercritical fluid extraction, however, have solid or satifactory antibacterial effects. The studies performed by Glišić *et al*. (2007), determined that the supercritical extract of carrot has low MIC values of 80, 160 and 640 μg/mL for the most of the investigated gram-positive bacteria, including *Bacillus* species, as well as for yeast. Accordingly, Mišić *et al*. (2008), obtained MIC values of 40 μg/mL for 2 *S.aureus* strains, and 320 μg/mL for 3 *S.aureus* and 6 *Listeria* strains when investigating antibacterial activity of supercritical extract of celery, which can be considered an excellent antibacterial effect.

In this study, however, the supercritical extracts of Common Mullein and Echinacea did not exhibit a solid antibacterial effect considering the high resulting MIC values of 2560 μg/mL or >2560 μg/mL for all tested bacterial strains. The results of antibacterial effects of supercritical extract of Angelica obtained in this study are similar to the results of previous studies conducted by Mišić *et al*. (2008), considering the resulting MIC values of 320 μg/mL and 640 μg/mL for the tested strains of *Staphylococcus*. As well as most publications of foreign and domestic authors, this paper clearly shows that the extracts obtained by supercritical fluid extraction, along with other plant extracts, have no effects on gram-negative bacteria.

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## ISPITIVANJE ANTIBAKTERIJSKOG DELOVANJA NATKRITIČNIH EKSTRAKATA BILJAKA KAO I EKSTRAKATA DOBIJENIH DRUGIM TEHNOLOŠKIM PROCESIMA NA NEKE BAKTERIJE IZOLOVANE OD ŽIVOTINJA

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SADRŽAJ

Multirezistencija na antibiotike bakterija kao i nedostatak novih antibiotika na tržištu lekova podstakao je ispitivanja antibakterijskog delovanja supstancija koje nisu antibiotici uključujući i biljne ekstrakte. Tokom prethodnih decenija, dokazano je da ekstrakti pojedinih biljaka imaju izraženo antibakterijsko delovanje ali je zbog prisustva tragova organskih rastvarača, njihova klinička upotreba ograničena. Ekstrakti biljaka dobijeni procesom natkritične ekstrakcije, međutim, nemaju tragove rastvarača, a na osnovu dosadašnjih ispitivanja je utvrđeno da deluju na neke gram-pozitivne bakterije. Ovim komparativinim ispitivanjem obuhvaćeni su ekstrakti divizme, anđelike i ehinacee dobijeni procesima natkritične

ekstrakcije, Soxlet-ove ekstrakcije i ultrazvučne ekstrakcije. Ispitivanje njihovog antibakterijskog delovanja vršeno je na nekim sojevima stafilokoka, *Enterobacter cloacae* i *E. coli* izolovanim iz uzoraka kliničkog materijala poreklom od životinja i ljudi. U ispitivanje je bio uključen i referentni soj *S. aureus* ATCC 25923. Za ispitivanje antibakterijskog delovanja ekstrakata primenjen je makrodilucioni metod u bujonu pomoću koga su određivane vrednosti MIC ekstrakata. Najjače antibakterijsko delovanje, odnosno najnižu vrednost MIC od 40 μg/mL za soj *S. epidermidis* imao je ekstrakt anđelike dobijen ultrazvučnom ekstrakcijom. Ekstrakt anđelike dobijen natkritičnom ekstrakcijom je takođe pokazao značajno antibakterijsko delovanje na sve sojeve stafilokoka obuhvaćenih ispitivanjem sa vrednostima MIC od 320 do 640 μg/mL. Ispitivanjem ekstrakata ehinacee i divizme dobijenih procesom nadkritične ekstrakcije kao i ekstrakta ehinacee dobijenog Soxlet-ovom ekstrakcijom, nije utvrđeno antibakterijsko delovanje jer je vrednost MIC ovih ekstrakata iznosila 2560 μg/mL ili >2560 μg/mL za sojeve bakterija koji su obuhvaćeni ispitivanjem.