ORIGINAL ARTICLE

STUDY ON ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF ETHANOLIC EXTRACT OF *HUMULUS LUPULUS*

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Abstract

The vegetal material is an inexhaustible source of biologically active compounds. It was already demonstrated that secondary metabolites of plant origin have practical application in the industry of food and beverage, drugs, and flavours. Furthermore, considering the acquired resistance of bacterial pathogens to existing antibiotics, research on new molecules of plant products is nowadays of great interest. Taking all these into consideration, we conducted a study on the inhibitory activity of *Humulus lupulus* L. (hops) extract against several gram negative and gram positive bacteria, (*Bacillus subtilis*, *Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus aureus, Enterobacter cloacae, Pseudomonas fluorescens* and *Escherichia coli).* The antimicrobial activity was performed by diffusion methods. The hops extract was also studied for its radical scavenging activity and total phenol content and total flavonoid content. The results obtained indicated that the hops extract could be used as raw material in the design of a plant based antimicrobial product.

Rezumat

Materialul vegetal reprezintă o sursă inepuizabilă de compuși biologic activi. A fost deja demonstrat faptul că metaboliții secundari de origine vegetală au aplicații în industria alimentară și în industria farmaceutică. În plus, având în vedere rezistența dobândită a agenților patogeni la antibioticele existente, cercetările asupra unor noi molecule provenind din produse vegetale sunt în prezent de mare interes. Luând toate acestea în considerare, am desfășurat un studiu privind activitatea inhibitoare a extractului din *Humulus lupulus* L. (hamei) împotriva unor bacterii gram negative și gram pozitive (*Bacillus subtilis, Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus, Enterobacter cloacae, Pseudomonas fluorescens și Escherichia coli*). Activitatea antimicrobiană a fost testată prin metoda disc-difuzimetrică. De asemenea, a fost analizat conținutul de polifenoli, flavonoide și activitatea antioxidantă a extractul din hamei. Rezultatele obținute au indicat faptul că extractul din hamei ar putea fi utilizat ca materie primă în proiectarea unui produs antimicrobian pe bază de plante.

Keywords: plant extracts, antibacterial activity, antioxidant activity, polyphenols, hops

Introduction

Plant extracts were used in folk medicine in the form of tinctures, infusions and decoctions or essential oils, as a cure and treatment of diseases all over the world. The most common studied plants for their therapeutic potential are considered the spices and medicinal herbs. Although they are extensively used for the design and development of new drugs in human medicine, plant antimicrobial compounds are also a promise for future plant disease controlling agents. The use of plant products as antimicrobial agents is an ancient idea [1], but the researches in the area are gaining attention lately. As a response to the acquired pathogens resistance to antibiotics existing on the market, new alternatives should be designed for the treatment of infectious diseases. It is therefore desirable to explore the potential of plant extracts for the development and design of new anti-microbial agents [2, 3], as this could be a solution for both medical and phytopharmaceutical industry. Plants contain natural bioactive compounds such as secondary metabolites and antioxidants. The medicinal plants used as traditional medicine all over the world are rich in secondary metabolites [4].

Hops (*Humulus lupulus*) are a dioecious perennial plant belonging to the genus *Humulus* in the family *Cannabaceae*, *Urticacales* order. The female inflorescences of *Humulus lupulus* (hops or hop) are well-known as bittering agents in the brewing industry [5]. Hop cones, presenting a high content of polyphenolic compounds and acyl phloro-glucides, are used in the manufacturing process to

preserve and to give beer its characteristic flavour [6, 7]. In addition to the application as a raw material in the brewing industry, hops have been used for a long time for various medical purposes such as sleep disturbances, insomnia anxiety, excitability and others [8-10]. Recent scientific studies confirm the use of hop extracts for treating acne, dysmenorrhea and amenorrhea [11].

In Romania's spontaneous flora, hops grow in shady areas near the rivers on the edge of forests, through groves, mostly uphill, through shady meadows or depressions in the lowlands. It is cultivated as crops or decorative plant. The largest fields' hops can be found in Transylvania, where the cones are valued for the brewing industry.

The aim of the present work was the study of the antibacterial activity of hops derived extract against several gram positive and gram negative bacteria such as *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Enterobacter cloacae*, and *Pseudomonas fluorescens*. The hops extract was also studied for the determination of its total polyphenols content and total flavonoid content and for the DPPH scavenging activity [30-32].

Materials and Methods

Chemical reagents

Mueller Hinton Agar (MHA)-Scharlab and Luria Bertani Agar (LBA) - Roth were used in the microbiological tests.

1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), Folin-Ciocalteu reagent, gallic acid and rutin reagents were purchased from Sigma Chemicals, while the sodium carbonate, aluminum chloride and potassium acetate were purchased from Fluka Chemical. All of the reagents and solvents used in the experiment were of adequate analytical grade.

The plant material and extraction

The sample plants were collected from fields located in the Southern part of Romania. The female inflorescences of *H. lupulus* were dried in a ventilated space, at room temperature, in shade. Plant materials were identified by researchers from the Biotechnologies Department from the National Institute of Research and Development for Biological Sciences and authenticated voucher specimens were deposited in the *Herbarium* of the same department.

In order to be used for extraction, the *Humulus lupulus* cones were grinded with a laboratory mill to a fine powder. Taking into consideration previous studies of the authors, regarding the antimicrobial activity and composition of other plant extracts (data not shown) the hydroalcoholic extracts were obtained by ultrasonication followed by maceration. Ultrasonication was performed at the following parameters: sonication time 30 minutes, temperature 35°C, power 34 watts. The maceration was performed in sealed recipients, for 96 hours, in dark, at room temperature, with occasional shaking. The solvent used was ethanol 70%, and the ratio between the vegetal material and the solvent was 1:10. The extract was first filtered through Whatman No.1 filter paper, and then sterilized by filtration using a 0.22 µm filter membrane. The resulting solution was kept at 4°C, in dark sealed bottles until further use in the microbiological studies.

Microbial Inoculum

The microbial strains used in the experiments presented in this paper are belonging to the Collection of the Biotechnologies Department, National Institute of Research and Development for Biological Sciences, Bucharest. The bacterial strains were maintained on Luria Bertani Agar and were as follows: *Bacillus subtilis*, *Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus aureus, Enterobacter cloacae, Pseudomonas fluorescens* and *Escherichia coli*.

Antimicrobial activity testing

The antibacterial activity was performed by the disc diffusion method. Firstly, the Mueller Hinton agar (MHA) plates were inoculated with the selected bacteria: *Bacillus subtilis*, *Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus aureus, Enterobacter cloacae, Pseudomonas fluorescens* and *Escherichia coli*. After that, the inoculum of the bacterial strains was prepared from fresh bacterial cultures (18 - 20 h old) and saline suspensions were adjusted to reach 0.5 McFarland standard turbidity, 100 µL of suspension containing 1.0×108 CFU mL^{-1} of bacteria. The plates were allowed to dry for 15 minutes and then 5 mm sterilized filter paper discs impregnated with the extract or with the positive and negative control were placed on the inoculated agar plates. The plates were incubated at 37 ± 2°C for a period of 24 hours. Negative controls were prepared with the solvent (ethylic alcohol 70%) and positive controls were antibiotics impregnated discs with Rifampicin 5 µg or Vancomycin 30 µg. Following this incubation, the radius of the inhibition zones around the discs was measured in millimetres. The experiment was performed in triplicate and the results were reported as mean ± standard deviation.

Determination of total phenolic content (TPC)

The total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu method. A dilute solution of ethanolic extract or gallic acid was mixed with Folin-Ciocalteu reagent and sodium carbonate (7%). The reaction mixture was allowed to stand for 30 minutes at room temperature and the absorbance of the samples was measured at 765 nm using a

UV/VIS spectrophotometer. The TPC of the extract was expressed as mg of gallic acid equivalent (GAE)/g dry weight (dw), using the equation obtained for the calibration curve of gallic acid prepared in various concentrations.

Determination of total flavonoid content (TFC)

The total flavonoid content was determined using the aluminium colorimetric method [12] with some modifications. Briefly, 0.5 mL of the extract or of the standard was added to 0.1 mL of aluminium chloride (10%) and 0.1 mL potassium acetate (1 M), and 2.8 mL distilled water and 1.5 mL methanol 80%. After incubation at room temperature for 30 de minutes, the absorbance of the reaction mixture was measured at 415 nm using an UV/VIS spectrophotometer. The TFC was expressed as mg of rutin equivalents (RE)/g dw, using the equation obtained for the standard curve of rutin prepared in various concentrations.

Determination of DPPH free radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay of the hops extract was performed spectrophotometrically, as previously described by Blois *et al.* with some modifications [13]. Briefly, an appropriate quantity of the plant extract was mixed with 2 mL of DPPH solution (0.1 mM) in ethanol. The DPPH solution was freshly prepared and protected from light. The absorbance of the reaction mixture was measured at 515 nm after incubation at room temperature, in the dark, for a period of 30 minutes. The control contained all of the above components except the extract. The measurements were performed in triplicate and the mean value was reported.

The radical scavenging activity was calculated using the following equation:

$$AA_{DPPH}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ is the optical density of the control (containing all reagents except for the extract) and A_{sample} is the optical density in the presence of the extract.

The antioxidant properties were expressed as DPPH %. The extract concentration providing 50% of free radical scavenging activity (EC50) was calculated from the graph of the radical scavenging activity percentage against extract concentration. This coefficient, which measures the effective concentration at which the DPPH radicals were scavenged by 50%, was obtained by extrapolation from linear regression analysis [14].

Results and Discussion

Antimicrobial activity

After an incubation period of 24 hours, the inhibition area formed around the discs impregnated with plant extract was examined. Generally, the plant extract developed clear inhibition areas for most of the tested strains. Seven bacterial strains were investigated for their resistance on hydroalcoholic extract obtained from *Humulus lupulus* (hops) female inflorescences.

The strongest antibacterial activity, demonstrated by the inhibition area, was observed against *B. subtilis* and *S. aureus* (Figure 1). The inhibition area of the hops extract against the growth of *B. subtilis* strain was equal to the antibiotic control used, namely vancomycin (30 µg). For *S. aureus* the inhibition radius was lower than for *B. subtilis*, but still close to the value determined for the positive control.

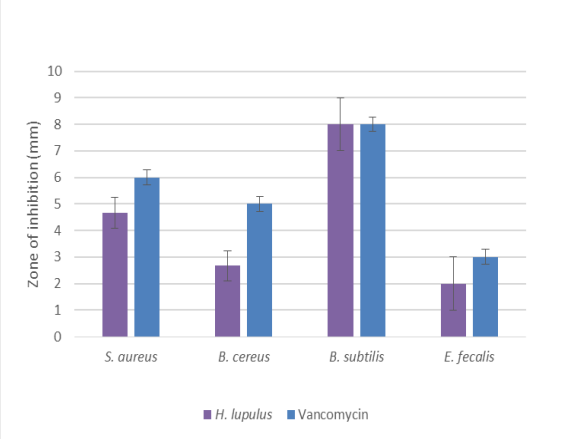
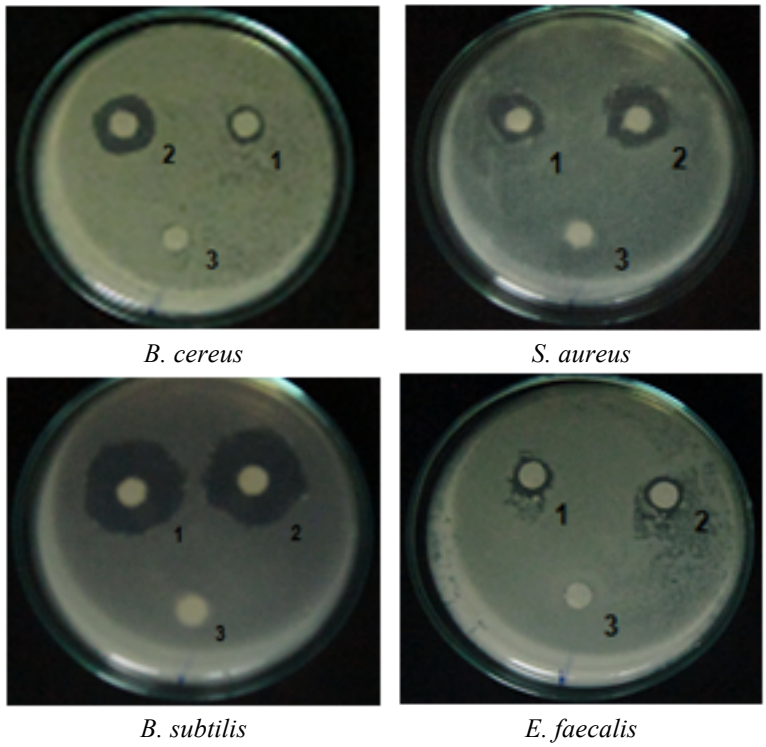


Figure 1.
The effect of hops ethanolic extract on gram positive bacterial strains (results are presented as the mean of triplicate determinations ± standard deviation)

The results observed for *B. cereus* strain were much lower, with a smaller inhibition halo, than for *B. subtilis* and *S. aureus*. However, it can be considered that the hops extract has an antibacterial activity against this strain. Nevertheless, one of the most resistant bacterial isolate proved to be *E. faecalis*, with an inhibition radius of only 2 mm (Figure 2).

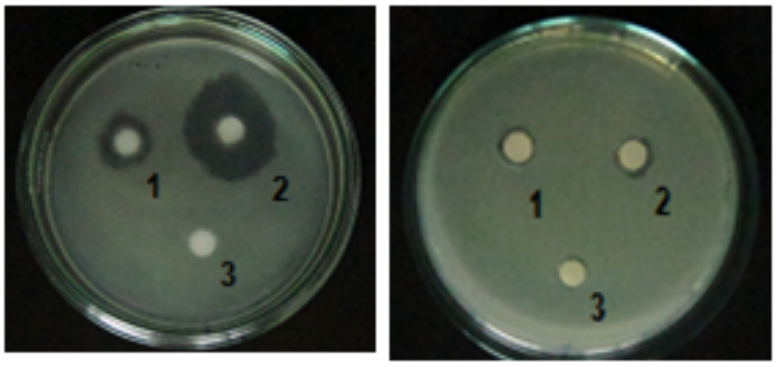


B. cereus *S. aureus*

B. subtilis *E. faecalis*

Figure 2.
The antimicrobial activity tests on gram positive bacteria
(1 - *H. lupulus* extract; 2 - positive control (antibiotic); 3 - negative control (ethylic alcohol))

Although it was not the best result obtained in this screening, it was observed that the tested extract inhibited the growth of *E. cloacae* more than the antibiotic control used, namely rifampicin (5 μg), with an inhibition area of 2.66 mm, *vs.* 2 mm for the antibiotic (Figure 3).



E. coli *E. cloacae*

Figure 3.
The antimicrobial activity tests on gram negative bacteria
(1 - *H. lupulus* extract; 2 - positive control (antibiotic); 3 - negative control (ethylic alcohol))

However, it was observed that the hops extract had no inhibition effect on the growth of *Pseudomonas fluorescens* strain (Figure 4).

Reporting the results obtained regarding the inhibition halo of the hops extracts *vs.* the effect of antibiotic substance used in this experiment, it was observed that the extract of hops reached the same intensity effect with the antibiotic (vancomycin 30 mg) when considering *B. subtilis* strain. In the case of *E. faecalis*, the extract of hops presented an efficiency of 66%. The only value below 50% was calculated with hops extract against the strain of *E. coli* for which the antibiotic had a maximum effect. Therefore, the antibacterial activity testing of the hops hydroalcoholic extract showed that most of the microbial strains taken into consideration were inhibited by the extract. The results obtained for the vegetal extracts of hops are promising and may

provide a basis for in-depth studies on the chemical composition of these extracts, mechanism of action against target bacteria and cytotoxicity effects.

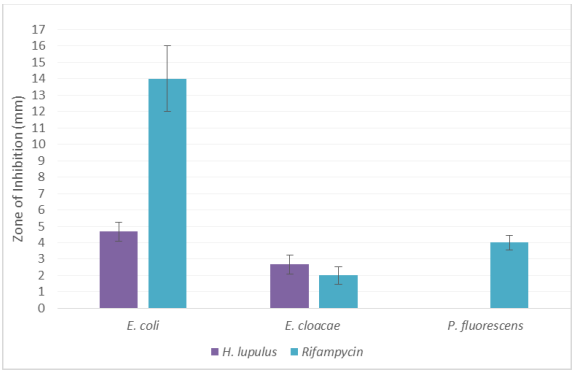


Figure 4.
The effect of hops ethanolic extract on gram negative bacterial strains (results are presented as the mean of triplicate determinations ± standard deviation)

These results are in accordance with the ones reported by Kramer et al, who found that gram-positive bacteria were strongly inhibited by hop extracts containing β-acids and xanthohumol, whereas the gram-negative bacteria were highly resistant against all tested hops extracts [15]. Similar results were reported by other authors regarding the ethanol extract of hops whole plant (stems, leaves and roots). The extract of hops displayed strong inhibitory effects against gram positive bacteria including *Staphylococcus aureus* (20.52 mm diameter of inhibition zone) and *Bacillus subtilis* (22.52 mm). Moreover, the extract was active against *Mycobacterium tuberculosis*, a pathogen that has been recognized as being involved severe pathologies worldwide. The hops extract had a weak activity against gram negative bacteria such as *E. coli* and *Pseudomonas aeruginosa* [16].

Recent scientific studies confirm the use of hops extracts for treating acne, dysmenorrhea and amenorrhea. Moreover, it was demonstrated that the hops extract presents antioxitocine properties [17]. Hops can also be used in the manufacture of poultice. Its antibacterial qualities stimulate the production of gastric fluid. On the basis of the ethno medical data on this plant, there were conducted studies on its antibacterial effect against gram-positive bacteria. The ethanol extract of *H. lupulus* showed antibacterial activity on *B. subtilis* and *S. aureus* [18].

In the same time, the study undertaken by Shapouri and Rahnema showed clearly that extracts of hops cones, were effective against *in vitro* growth of *Brucella abortus* and *Brucella melitensis* in cell culture of mice macrophages. The inhibitory effect of hops aqueous, acetonic and ethanolic extracts shown on *B. abortus* 544 and *B. melitensis* 16M were studied by both broth macrodilution and agar-well diffusion methods. The alcoholic extract showed the most intense inhibitory effects [19].

Plant extracts characterization

The antimicrobial activity of extracts obtained from vegetal material is generally attributed to the content of biologically active compounds present in the plant. It is widely considered that the anti-bacterial activity of plant extracts is related to the polyphenol content [20]. Therefore, besides their role as antioxidants, polyphenols (especially flavonoids) have antimicrobial properties. Plant phenolics include from simple, low molecular weight compounds to large and complex polyphenols. The main phenolic compounds from plants can be classified into 2 groups: flavonoids (polyphenolic compounds comprising 15 carbons and 2 aromatic rings connected with a 3 carbon bridge) and non-flavonoids (phenolic acids, tannins, stilbenes, etc.). The scientific studies highlighted many important biological activities of the plant phenolics, such as antimicrobial, allelopathic, antioxidant, anti-inflammatory, cardio-protective, anti-mutagenic, anticarcinogenic and anti-ageing properties. Also, they contribute to inducing apoptosis by different pathways, like arresting cell cycle, migration, proliferation or differentiation, regulating carcinogen metabolism, inhibiting cell adhesion, blocking signalling pathways and others [21, 22].

The total phenolic content (TPC) of the hops extract was determined spectrophotometrically based on the calibration curve of gallic acid ($y = 0.0047x + 0.0563$, with a regression coefficient $R^2 = 0.993$). The total flavonoid content (TFC) expressed as mg rutin equivalents/g dw was determined based on the calibration curve of rutin ($y = 0.022x + 0.039$, with a regression coefficient $R^2 = 0.991$). The data reported represent the results of triplicate experiments. The results obtained for the hops extract are presented in Figure 5.

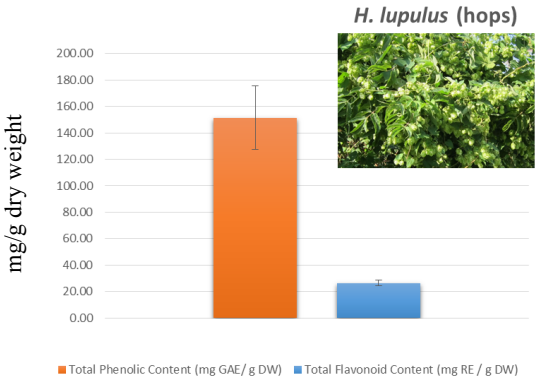


Figure 5.
The total phenolic content and the total flavonoid content of hops extract

A rapid method for measuring antioxidant capacity involves the use of free radical 1,1- diphenyl-2-picrilhidrazil (DPPH) [23, 24]. Usually, EC50 is used to compare the anti-radical activity that refers to a concentration of the extract at which is scavenged 50% of DPPH radicals. The results obtained provide a direct correlation between the values obtained for EC50 factor and for the total phenolic and flavonoid content (Table I). Therefore, the lower this value is, the higher the anti-radical effect is.

Table I

The total phenolic content, the total flavonoid content and antioxidant activity EC50 of *Humulus lupulus* extract

TPC (mg GAE/g dw)	TFC (mg RE/g dw)	DPPH scavenging activity EC50 (µg/mL)
151.42 ± 23.97	26.46 ± 2.03	83.808 ± 3.57

*Results are presented as the mean of triplicate determinations ± standard deviation

The results obtained regarding the DPPH radical activity of the ethanolic extract obtained from *Humulus lupulus* collected from Romania are presented in Figure 6.

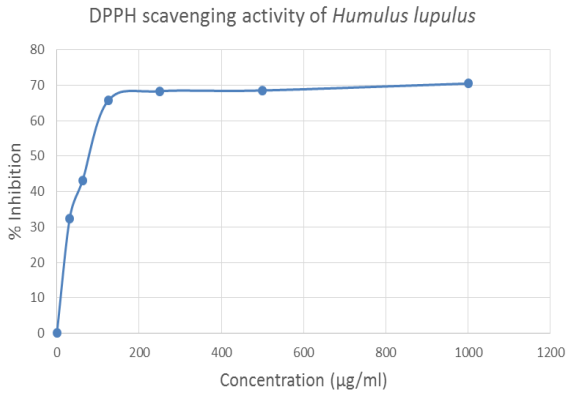


Figure 6.
DPPH scavenging activity of *Humulus lupulus* (results are presented as the mean of triplicate determinations)

The results of this paper are in accordance to those of other reports, demonstrating that plant extracts with high levels of total phenolic content have good antioxidant potential. Previous studies found that antioxidant activity of plant extracts is positively correlated with total phenolics content [25, 26], while others have found no such relationships [27 - 29]. Plant extracts have a long history in traditional medicine. They have been used in human medicine in the form of tinctures, infusions and decoctions or essential oils. Secondary plant metabolites is a topical issue, those being studied as effective sources in the development of new drugs for use in human and veterinary medicine. Antimicrobial plant compounds can be considered an important source of raw material in the development of antimicrobial products.

Conclusions

The extract obtained from dried hops flowers showed good antimicrobial activity against most of the tested strains. The results indicated that this plant considered mainly as raw material in brewing industry has antibacterial and antioxidant potential. The chemical analysis revealed considerable levels of polyphenols with appreciable amounts of flavonoids. Chemical compounds derived from hops are a good source of biologically active substances. Therefore, it may have future practical applications in the development of an antimicrobial product based on natural compounds, with multiple uses in industry. Nevertheless, further studies are needed to characterize the chemical composition and structures that contribute to the antimicrobial and antioxidant activity of hops extracts.

Acknowledgements

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