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ASSESSMENT OF THE THERAPEUTIC POTENTIAL APPLICATION OF THE EXTRACTS FROM DIOECIOUS HEMP (*Cannabis sativa* L.)

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Abstract

Dioecious hemp (Cannabis sativa L.) is an industrial crop utilised for various purposes, including fibre, seeds, and plant extracts. The seeds and plant extracts are used for food, pharmaceuticals, or cosmetic products. This study explores the therapeutic potential of dioecious hemp by analysing the antibacterial activity of hydroalcoholic extracts from four varieties of CS: CS 1 - Armanca, CS 2 - Lovrin 110, CS 3 - Silvana, and CS 4 - Teodora. The antibacterial properties of the examined extracts were evaluated against two clinically significant ATCC bacterial strains: Staphylococcus aureus (ATCC 25923) and Escherichia coli (ATCC 25922). The main parameters assessed included bacterial growth percentage (BGP%) and bacterial inhibition percentage (BIP%), and minimum inhibitory concentration (MIC). These were utilised to evaluate the effectiveness of the extracts. The findings indicated a positive correlation between the concentration of CS extract and its antibacterial activity. Specifically, higher extract concentrations resulted in stronger inhibitory effects on bacterial growth. However, further research is crucial to fully understand the potential of hemp hydroalcoholic extracts as components in antibacterial pharmaceutical products.

Key words: *dioecious hemp extracts, antibacterial activity, BGP, BIP, MIC, hydroalcoholic extracts.*

INTRODUCTION

Dioecious hemp (*Cannabis sativa* L.) is a versatile plant prized for its industrial, nutritional, and therapeutic uses (Andre et al., 2016). As a dioecious species, *C. sativa* produces separate male and female plants, each contributing uniquely to its chemical profile (Small et al., 2007). Cannabis is extensively grown globally and has a rich cultivation history in China, valued for its uses in textiles and consumption. Its historical uses for fibres, seeds, and medicinal properties, as well as contemporary studies concentrating on its bioactive compounds, connect us to a rich heritage (Gallily et al., 2015).

The pharmacological potential of *C. sativa* arises from its complex chemical composition, which encompasses over 565 natural compounds, prominently 120 cannabinoids along with terpenoids and flavonoids (ElSohly & Slade, 2005). Cannabinoids, particularly cannabidiol (CBD) and tetrahydrocannabinol (THC), demonstrate diverse biological activities, including antimicrobial effects (Appendino et al., 2008). A recent review highlighted the uses of *C. sativa* from ancient times to today, covering ethnological, botanical, chemical, and pharmacological aspects, emphasising pharmaceutical research to illustrate the plant's potential therapeutic benefits (Bonini et al., 2018). Particularly, the morphological components, like the inflorescence, are crucial because they contain potentially biologically active compounds (Richins et al., 2018; Tiago et al., 2022).

As antimicrobial resistance continues to rise globally, *C. sativa* extracts are gaining interest for their antibacterial effects. Research demonstrates the effectiveness of hydroalcoholic extracts against *Staphylococcus aureus* and *Escherichia coli*, showing that inhibitory activity increases with higher extract concentrations (Blázquez & Bermejo, 2018). Moreover, root extracts from *C. sativa* demonstrate antioxidant and bactericidal effects, reinforcing its therapeutic importance (Pollastro et al., 2018). *E. coli* poses a significant threat to the healthy growth of both humans and animals (Lagha et al., 2019). In certain circumstances, it can lead to diarrhea in humans and animals (Yue et al., 2020). Additionally, it can cause food

spoilage, which is a central concern in food preservation (Gutiérrez-del-Río et al., 2018). *S. aureus* is a Gram-positive, aerobic bacterium that acts as an opportunistic pathogen (Zamora-Mendoza et al., 2022). This bacterium primarily colonises the skin and mucous membranes. It can lead to diseases ranging from common skin infections to more severe conditions such as endocarditis, bloodstream infections, and osteomyelitis (Jenul & Horswill, 2019).

Numerous studies indicate cannabidiol (CBD) may possess antimicrobial properties, particularly against drug-resistant *S. aureus* strains (Martinenghi et al., 2020; Ferenczy et al., 1958; Appendino et al., 2008). Both cannabis extracts and isolated cannabinoids have demonstrated greater effectiveness against Gram-positive bacteria, including multidrug-resistant species (Schofs et al., 2021). Although there are promising results, the antimicrobial capabilities of certain *C. sativa* varieties have still not been thoroughly researched. This study assesses the antibacterial effects of hydroalcoholic extracts from four dioecious varieties against *S. aureus* and *E. coli*, aiming to aid in the creation of new antibacterial agents derived from *C. sativa*.

MATERIALS AND METHODS

The study was conducted as a field experiment during the 2023 growing season at the Agricultural Research Station of University of Life Sciences "King Mihai I" from Timisoara, Romania. This location was chosen due to its conducive climate and soil conditions for hemp cultivation, providing an ideal setting for the study. A randomised block design utilised four dioecious *Cannabis sativa* L. varieties as the experimental factor: CS1 - Armanca, CS2 - Lovrin 110, CS3 - Silvana, and CS4 - Teodora. Each variety was replicated three times, with plots measuring 10 m² (Figure 1). Rows were spaced 70 cm apart, while plants in a row were 50 cm apart. To promote optimal growth conditions, seeds were sown manually. Uniform agronomic methods were applied across all plots. Female inflorescences were harvested at full bloom. The collected material was air-dried in a shaded area at room temperature (around 22°C) to maintain bioactive compounds. Using a laboratory mill, the dried material was then ground into a fine powder. For extraction, 1 g of the plant material is mixed with 10 ml of 70% ethanol for 30 minutes with vigorous shaking. The resulting extracts are filtered through high porosity filter paper. After extraction, the samples were diluted to a ratio of 1:100 with 70% ethanol.

Figure 1. Four dioecious *Cannabis sativa* L. varieties

Microbiological method: A 10^{-3} dilution of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) strains were used. The cultures were then diluted to an optical density (OD) of 0.5 McFarland standard (1,5 × 10^8UFC×mL) using Brain Heart Infusion (BHI) broth (Oxoid, CM1135) and assessed with a McFarland densitometer (Grand-Bio, England). Subsequently, 100 µl of the dilutions were

pipetted into each well of a 96-well microdilution plate using a Calibra 852 digital multichannel pipette.

Test extracts were added in volumes of 25 µL, 50 µL, 75 µL, and 100 µl. The plates were covered and incubated for 24 hours at 37°C. After this period, the optical density (OD) was measured at 540 nm using an ELISA reader (BIORAD PR 1100, Hercules, CA, USA). All samples were tested in triplicate. For the positive control, strain suspensions in BHI were utilised. The broth microdilution method is a fundamental technique for antimicrobial susceptibility testing (Clinical and Laboratory Standards Institute, 2017, Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically: Approved Standard-8th Edition, CLSI Document M07-A8, Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2009. Medicines, 4, 58). This method involves double dilutions of the antimicrobial agent in a liquid culture medium, which is organised in 96-well microtiter plates. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that prevents the growth of the organism. CLSI has standardised the broth microdilution method for aerobically growing bacteria, yeasts, and filamentous fungi.

The EUCAST microdilution method closely resembles the CLSI, but it usually includes modifications related to specific test parameters like inoculum preparation, size, and MIC reading.

RESULTS AND DISCUSSIONS

Results are shown as bacterial growth percentage (BGP%) and bacterial inhibition percentage (BIP%). These percentages are calculated using formulas (1) and (2):

$$BGP\% = OD_{sample}/OD_{negative\ control} \times 100 \quad (1)$$

$$BIP\% = 100 - BGP \quad (2)$$

In all tested extracts, bacterial growth percentages presented a negative correlation with the increasing concentration tested as presented in Figure 2 and Figure 3.

Figure 2 presents a graphical representation of the efficacy of CS extracts against *S. aureus* expressed as BGP% and BIP% while Figure 3, represents graphically the activity of CS extracts against *E. coli*.

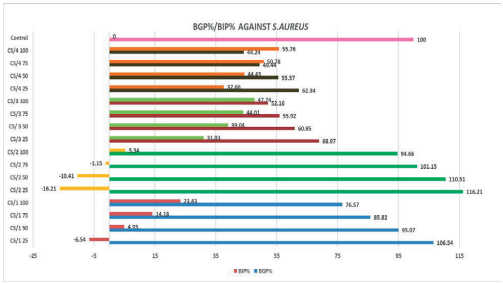

Figure 2. Graphical representation of the activity of CS extracts against *Staphylococcus aureus* expressed as bacterial growth percentage (BGP %) and bacterial inhibition percentage (BIP %)

The antibacterial activity of CS1 - Armanca extract is mild to moderate and rises with increasing dosage.

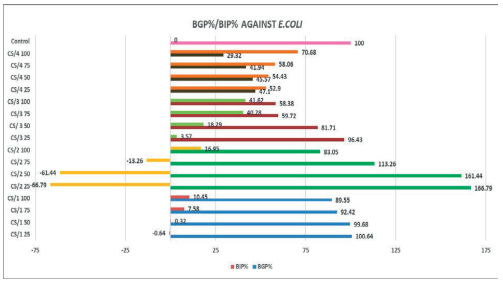

Figure 3. Graphical representation of the activity of CS extracts against *Escherichia coli* expressed as bacterial growth percentage (BGP %) and bacterial inhibition percentage (BIP %)

When it comes to *S. aureus*, the bacterial growth percentage (BGP %) decreases from 106.54% at 25 µL to 76.57% at 100 µL, while the bacterial inhibition percentage (BIP %) increases from -6.54% to 23.43%. This indicates an inhibitory effect that is dose-dependent. While less noticeable, a similar pattern is seen when looking at *E. coli*, BIP % increases from -0.64% to 10.45%, while BGP % decreases from 100.64% to 89.55%. Slightly more effective against *S. aureus* than *E. coli*, CS1 - Armanca exhibits a moderate inhibitory effect overall.

Considering the bacterial growth CS2 - Lovrin 110 exhibits inconsistent and generally weak antibacterial effects. At lower doses (116.21% at 25 µL), the BGP% for *S. aureus* exceeds 100%, suggesting enhanced bacterial growth as opposed to inhibition. The BGP % only decreases below 100% at 100 µL, when it reaches 94.66% (BIP % = 5.34%). The impact

on *E. coli* is more problematic: at 25 µL, BGP % raises significantly to 166.79% and remains higher than 100% up until the highest dose, at which point it decreases to 83.05% (BIP % = 16.95%). These findings suggest that CS2 - Lovrin 110 may only slightly inhibit bacterial growth at higher volumes and could actually stimulate bacterial growth at lower doses, especially for *E. coli*.

Strong antibacterial activity is shown by CS3 - Silvana, which steadily becomes more effective as the volume increases. BIP% values for *S. aureus* increase from 31.03% to 47.74%, while BGP % decreases from 68.97% at 25 µL to 52.16% at 100 µL. Similarly positive outcomes are observed for *E. coli:* BIP % increases from 3.57% to 41.62% while BGP % decreases from 96.43% to 58.38%. CS3 - Silvana is a highly effective treatment, especially noticeable for its balanced action against both *S. aureus* and *E. coli*. These results indicate an effective, dose-dependent antibacterial effect against both bacterial strains.

Among the tested samples, CS4 - Teodora has the strongest antibacterial activity, especially against *E. coli*. Strong inhibition is indicated according to the BGP % for *S. aureus*, which decreases from 62.34% at 25 µL to 44.24% at 100 µL (BIP % = 37.66% to 55.76%). The effect is even more noticeable against *E. coli*: BIP % ranges from 52.9% to 70.68%, while BGP% decreases from 47.1% to 29.32%. The data conclusively demonstrate that CS4 - Teodora inhibits bacterial growth in a potent, dose-dependent manner. It is particularly effective against *E. coli*, making it the most promising formulation tested for antibacterial use.

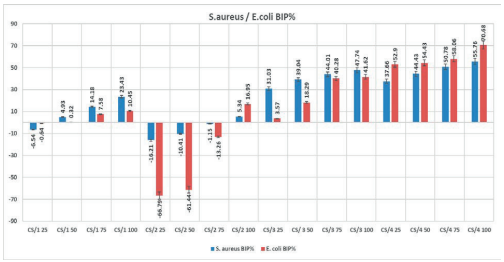

Figure 4. Graphical comparison of the CS efficacy against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922)

Figure 4 displays the inhibition values of CS extracts against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) strains in comparison and Table 1 presents a summary of the CS efficacy at the highest concentration tested.

Table 1. Summary of the CS efficacy at the highest concentration tested

Sample	BIP% *S. aureus ATCC 25923*	BIP% *E. coli ATCC 25922*	Most Effective Against
CS/1	23.43	10.45	*S. aureus*
CS/2	5.34	16.95	Weak/Inconsistent
CS/3	47.74	41.62	Both, slightly *S. aureus*
CS/4	55.76	70.68	*E. coli*

BIP%-bacterial inhibition percentage

CS4 - Teodora proved to be the most effective antibacterial formulation according to the inhibition percentages at the highest tested volume (100 µL). It was especially effective against *E. coli* (BIP % = 70.68%) and also significantly inhibited *S. aureus* (BIP % = 55.76%). With significant inhibition of both strains (*S. aureus* = 47.74%, *E. coli* = 41.62%), CS3 - Silvana came in second. While CS2 - Lovrin 110 was the least effective and even stimulated bacterial growth at lower doses, particularly in *E. coli*. CS1 - Armanca demonstrated moderate inhibition and was more effective against *S. aureus* (23.43%) than *E. coli* (10.45%). This suggests that *S. aureus* is partially more responsive to treatment at moderate concentrations, whereas *E. coli* could require higher doses for noticeable inhibition.

The findings indicate a concentration-dependent antibacterial effect, with higher extract concentrations associated with increased bacterial inhibition rates (BIP %) and decreased bacterial growth rates (BGP %). Blázquez and Bermejo (2018) reported similar findings, emphasising the effectiveness of cannabinoids against resistant bacterial strains. The inhibitory activity observed can likely be attributed to cannabinoids and terpenoids in the extracts, which disrupt bacterial membranes and inhibit biofilm development (Appendino et al., 2008). Notably, *S. aureus*, a Gram-positive bacterium, showed greater susceptibility to the extracts than *E. coli*, a Gram-negative bacterium. This aligns with Pollastro et al. (2018), which connects bacterial cell wall composition with different sensitivities to plant-derived antimicrobials.

Additionally, the minimum inhibitory concentration (MIC) values in this study fall within the range documented for hydroalcoholic extracts of *C. sativa* (Russo, 2011), highlighting their potential as natural antibacterial agents. The intense activity of CS3 - Silvana and CS4- Teodora corresponds with ElSohly and Slade (2005), who observed that specific hemp cultivars display varying bioactivity due to differences in their phytochemical compositions. The analysis reveals that dioecious hemp plant extracts exhibit a negative correlation with the tested concentration increase and the bacterial growth cofactors.

CONCLUSIONS

In conclusion, the antibacterial effectiveness of the tested samples varied significantly across both bacterial strains. CS4 - Teodora demonstrated the strongest inhibitory activity, followed closely by CS3 - Silvana, with both showing clear dose-dependent effects. CS1 - Armanca exhibited moderate activity, while CS2 - Lovrin 110 was largely ineffective and sometimes stimulated bacterial growth. Overall, the treatments were generally more effective against *S. aureus* at lower concentrations, whereas *E. coli* required higher doses for comparable inhibition. These findings imply that various hemp varieties may possess unique antibacterial mechanisms and efficacies depending on the concentration of the extract. In summary, this research emphasises the therapeutic promise of dioecious hemp as a source of bioactive compounds that possess antibacterial qualities. The findings lay the groundwork for further investigation into creating hemp-derived antibacterial products and stress the necessity of choosing particular hemp varieties and extract concentrations to enhance effectiveness. Subsequent research should aim to clarify the underlying mechanisms of action and investigate more uses for these natural extracts in fighting bacterial infections.

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