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Antibacterial Effect of *Allium sativum* L. and *Allium cepa* L. Extracts against Multidrug-Resistant *Escherichia coli* Strains Isolated from Broiler Chickens

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ABSTRACT

Over the past decades, the incidence of avian colibacillosis caused by multidrug-resistant *Escherichia coli* (*E. coli*) has increased dramatically worldwide. The present *in vitro* study focused on evaluating the antibacterial properties of *Allium sativum* L. and *Allium cepa* L. extracts against multidrug-resistant *E. coli* strains isolated from broiler chickens suffering from colibacillosis. The confirmation of *E. coli* isolates and their antibiotic resistance was performed using conventional methods. Furthermore, the antimicrobial activity of both extracts was assessed through the disk diffusion method, along with the determination of the minimum inhibitory concentration (MIC) via liquid macrodilution and the minimum bactericidal concentration (MBC) using solid media. The obtained results showed that the multidrug-resistant *E. coli* strains were extremely sensitive to garlic extract with a MIC of 41.5 mg / mL and CMB of 166 mg / mL and very sensitive to the combination of garlic and onion extracts. However, onion extract was ineffective against the resistant *E. coli* strains. The findings of the present study suggested the possibility of using garlic as an alternative to antibiotics in the treatment of colibacillosis caused by resistant *E. coli* strains.

Keywords: *Allium cepa* L., *Allium sativum* L., Broiler chicken, Colibacillosis, *Escherichia coli*, Multidrug-resistance

INTRODUCTION

Avian colibacillosis is an infectious disease caused by the bacterium *Escherichia coli (E. coli)*. This prevalent disease is a primary concern in the poultry industry worldwide, frequently leading to condemnation at slaughterhouses (Nolan et al., 2013; Naoufal et al., 2017). The disease significantly affects the health and welfare of poultry populations, presenting in various clinical forms ranging from acute septicemia to chronic respiratory and genital problems (Joseph et al., 2023). This bacterial infection causes substantial economic losses due to reduced productivity, higher mortality rates, and the need for extensive therapeutic treatments. *E. coli* is commonly found in the avian digestive system as a commensal organism, with most strains displaying non-pathogenic characteristics. However, certain strains, identified as Avian Pathogenic *E. coli* and belonging to specific serotypes, particularly O1, O2, and O78, are associated with the manifestation of the disease (Apostolakos et al., 2021; Joseph et al., 2023).

The management of this disease relies mainly on antibiotics. Although this approach has helped in combating harmful bacteria, it has also led to the development of antibiotic-resistant strains (Merati et al., 2020; Abdel-Rahman et al., 2023). The rise of antibiotic-resistant strains of *E. coli* makes treatment more challenging and raises concerns about the ongoing effectiveness of antimicrobial measures in poultry farming. Excessive antibiotic use has resulted in the development of multi-drug-resistant strains, highlighting the necessity of alternative methods to mitigate the effects of colibacillosis on both poultry health and industry viability (Bhattarai et al., 2024).

The emergence of antibiotic resistance poses a significant challenge in humans and veterinary medicine, often leading to treatment failures. Health authorities are particularly concerned about the rise of multi-resistant bacteria in community infections. Consequently, the scientific community has been actively researching alternative strategies to replace antibiotics and effectively manage colibacillosis in broiler farms (Koutsianos et al., 2021; Eid et al., 2022; Song et al., 2023).

Medicinal plants currently represent an interesting alternative to antibiotics; some plant extracts exhibit potent antimicrobial, fungicidal, and antiviral effects (Bhardwaj et al., 2016; Reiter et al., 2020; Abdallah et al., 2023). *Allium sativum* L., commonly known as garlic, and *Allium cepa* L., commonly known as onion, are bulbous herbaceous plants of the Alliaceae family. They are known to have a wide range of pharmacological properties, including antimicrobial, anticancer, and antioxidant effects. These plants have been utilized for centuries to treat various health conditions (Krstin

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et al., 2018; Raj et al., 2021; Oyawoye et al., 2022). Recent studies have highlighted the significant potential of garlic and onion as antimicrobial agents, emphasizing the importance of further research into their effectiveness against various multidrug-resistant clinical bacteria. The present study was conducted to evaluate the antibacterial activity of *Allium sativum* L. and *Allium cepa* L. extracts against multidrug-resistant *Escherichia coli* strains isolated from broiler chickens affected by colibacillosis.

MATERIALS AND METHODS

Ethical approval

This research was conducted *in vitro* by the guidelines of the Veterinary Sciences Institute, University of Tiaret, Algeria.

Plant material

The plants used in this study were purchased in October 2022 from a local market in Tiaret Province, Algeria. The white garlic variety was chosen for *Allium sativum* L., and the red onion variety was selected for *Allium cepa* L. The fresh plants were placed in bags and transported to the Laboratory of Hygiene and Animal Pathology, Tiaret, Algeria, within 24 hours.

Bacterial isolates

The antibacterial assays were carried out on five clinical multidrug-resistant *E. coli* strains, isolated by Merati et al. (2020) from the liver and spleen of broiler chickens exhibiting gross lesions suspected to be colibacillosis. The bacterial isolates were stored at -20°C in the Laboratory of Hygiene and Animal Pathology, Tiaret, Algeria.

Preparation of crude garlic (*Allium sativum* L.) and onion (*Allium cepa* L.) extracts

Fresh garlic and onion bulbs were washed and peeled, and then 50 g of each plant was weighed before being crushed in a sterile mortar and pestle under aseptic conditions. The resulting homogenized mixtures were centrifuged at 3000 rpm for 15 minutes, and the supernatants were filtered through Whatman 125 mm filter paper. The obtained extracts were designated as 100% concentration. The concentration was determined based on the total weight of the plant material per milliliter of extract. From 50 g of raw garlic, 12 ml of extract was obtained, corresponding to a concentration of 4.16 g/ml. Similarly, 50 g of raw onion produced 15 ml of extract, with a concentration of 3.33 g/ml. Before use, the extracts were inoculated onto nutrient agar (Biokar, France) and incubated at 37°C for 24 hours to ensure sterility (Yadav et al., 2015).

Revival of *Escherichia coli* isolates

The conserved bacterial isolates were inoculated into a tube containing peptone broth (Biokar, France) and incubated aerobically at 37°C for 24 hours to check their viability. These suspensions were then plated onto eosin methylene bleu (EMB) agar (Biokar, France) and incubated aerobically at 37°C for 24 hours. Colonies with a greenish metallic sheen were subcultured and plated onto EMB agar for purification. Pure colonies were stored at 4°C for confirmation of the bacterial isolates (Quinn et al., 2002).

Confirmation of *Escherichia coli* isolates

After purification of the colonies on EMB agar, they were confirmed as *E. coli* by various tests, including Gram staining, catalase test, oxidase test, and analysis of biochemical characteristics using Analytic Profile Index (API) 20 E test strips (BioMerieux, France; Quinn et al., 2002).

Susceptibility to antibiotics of *Escherichia coli* isolates

The antibiotic sensitivity test was performed using the standard disk diffusion method on solid media according to the recommendations of CASFM/EUCAST (2020). Mueller Hinton agar (MH; Biokar, France) and a range of antibiotics from different classes, in the form of discs impregnated with each molecule, were used. The present study included the following antibiotics Amoxicillin/Clavulanic acid (AMC: 20 µg; CYPRESS DIAGNOSTICS, Belgium); Tetracycline (TE: 30 µg; CYPRESS DIAGNOSTICS, Belgium); Nalidixic acid (NA: 30 µg; Liofilchem, Italy); Erythromycin (E: 15 µg; Liofilchem, Italy); and Ampicillin (AM: 10 µg; Bio-analyse, Turkey).

Assessment of the antibacterial activity of garlic and onion extracts

The agar diffusion technique

From a pure bacterial culture, four to five colonies were collected using a wire loop and emulsified in 5 mL of physiological saline. The resulting suspension was then adjusted to achieve a turbidity equivalent to 0.5 McFarland units (10^8 CFU/mL). The culture was streaked on MH agar plates using a sterile cotton swab and allowed to dry for about 5 minutes. On the surface of each plate, sterile Whatman paper discs, 6 mm in diameter, containing 10 µl of garlic extract, 10 µl of onion extract, and 10 µl of a 50/50 mixture of the two plant extracts were applied. During the process, two controls were performed: A negative control with a disc containing 10 µL of sterile distilled water, and a positive control with an antibiotic disc containing Amikacin (AMK, 30 µg; CYPRESS DIAGNOSTICS, Belgium). The plates were left

for one hour at room temperature for proper diffusion and then incubated in an upright position at 37°C for 24 hours. The test was performed in triplicate under the same experimental conditions, and mean of the diameter of inhibition zones was calculated (CASFM/EUCAST, 2020).

Determination of minimal inhibitory concentration

The determination of minimal inhibitory concentration was carried out by adding 400 µL of the crude plant extracts to be tested into a sterile tube containing 4.6 mL of MH Broth (MHB) medium (Biokar, France). A serial dilution was conducted in MHB medium to achieve concentrations of garlic ranging from 332 mg/mL to 1.29 mg/mL and concentrations of onion ranging from 266 mg/mL to 1.03 mg/mL. To each tube, 13 µL of bacterial inoculum, corresponding to a density equivalent to the 0.5 McFarland standard, was added. A bacterial growth control, in which 13 µL of standardized inoculum was added to the MHB medium, was also performed. After 48 hours of incubation at 37°C, the tubes were centrifuged at 5000 rpm for 5 minutes, and the minimal inhibitory concentration (MIC) was determined from the first tube in the series in which no bacterial growth was observed (Forbes et al., 1998; NCCLS, 2003).

Determination of minimal bactericidal concentration

The same concentration range utilized in the liquid macrodilution technique was applied to determine the minimal bactericidal concentration (MBC) of the plant extracts. A loopful of culture was taken from the control tube and from each tube where no growth was observed after 48 hours of incubation at 37 °C, and it was inoculated onto the surface of MH agar. The plates were then incubated for 24 hours at 37°C, and the MBC was identified as the plate showing no colony formation (Forbes et al., 1998; NCCLS, 2003).

Statistical analysis

Descriptive statistics were performed to compare results. The data were analyzed using Microsoft Excel 2016 (USA). The data were expressed as mean ± standard deviation.

RESULTS

Revival and confirmation of *Escherichia coli* isolates

The confirmation of the *E coli* isolates was determined based on morphological and biochemical characteristics. The isolates produced typical deep purple colonies with a greenish metallic sheen. Biochemical characterization using the API 20 E system revealed that all isolates tested positive for o-nitrophenyl-β-D-galactopyranoside, lysine decarboxylase, ornithine decarboxylase, indole, Voges-Proskauer, mannose, glucose, sorbitol, rhamnose, melibiose, and arabinose. Conversely, the isolates tested negative for arginine dihydrolase, citrate, hydrogen sulfide H_2S production, urease, tryptophan deaminase, gelatinase, inositol, saccharose, and amygdalin.

Antibiotic susceptibility of *Escherichia coli* isolates

This test aimed to evaluate the antibiotic resistance of the *E. coli* isolates. The results of the antibiotic susceptibility test are shown in Table 1. According to the results of the antibiogram presented in Table 1, a complete absence of inhibition zones was observed around the five antibiotic discs tested involving amoxicillin + clavulanic acid, ampicillin, erythromycin, nalidixic acid, and tetracycline. These findings confirmed the resistance of the tested *E. coli* isolates in the present study.

Antibacterial activity of garlic and onion extracts

The antibacterial activity of the studied extracts was evaluated using the disc diffusion method on an MH agar medium (Figure 1). It was a qualitative technique based on determining the diameter of inhibition zones around discs loaded with the tested extracts. Table 2 presents the diameters of inhibition zones for garlic extracts, onion extracts, and their combination. According to the results presented in Table 2, the resistant strains of *E. coli* tested were sensitive to both garlic extract and the combination of garlic and onion extracts, with inhibition zones of 20.5 mm ± 1.29 and 16 mm ± 0.57, respectively. However, there was a complete absence of an inhibition zone for the onion extract, indicating that there was no antibacterial activity against the tested *E. coli* strains. The positive control confirmed that the bacteria were susceptible to amikacin, with an inhibition zone of 24.33 mm ± 0.47. The MIC was a quantitative technique that involved exposing a microorganism to progressively diluted concentrations of the test substance to determine its inhibitory effect. In the present study, only garlic exhibited antibacterial effects against the multidrug-resistant *E. coli* isolates tested, therefore, the MIC and MBC of the crude extract of *Allium sativum* L. were determined (Figure 2), and the results indicated that the MIC was 41.5 mg/mL, while the MBC was 166 mg/mL.

Table 1. Antibiotic sensitivity of isolated *Escherichia coli* from broiler chickens affected by colibacillosis

Antibiotics	Concentration of the disc	Inhibition zone	Results*
Amoxicillin + clavulanic acid (AMC)	20 µg	Absence	R
Ampicillin (AM)	10 µg	Absence	R
Erythromycin (E)	15 µg	Absence	R
Nalidixic acid (N)	30 µg	Absence	R
Tétracycline (TE)	30 µg	Absence	R

R: Resistant; * Recommendations of CASFM/EUCAST (2020).

Table 2. Antibacterial activity of the *Allium sativum L.* and *Allium cepa L.* extracts against isolated *Escherichia coli* from broiler chickens affected by colibacillosis

Tested extracts/antibiotic	Inhibition diameter (mm)	Sensitivity
Allium sativum	20.5 ± 1.29	S
Allium cepa	Absent	R
Allium sativum + *Allium cepa*	16.5 ± 0.57	S
Amikacin (AMK: 30 µg)	24,33 ± 0.47	S

R: Resistant; S: Sensitive; *Recommendations of CASFM/EUCAST (2020).

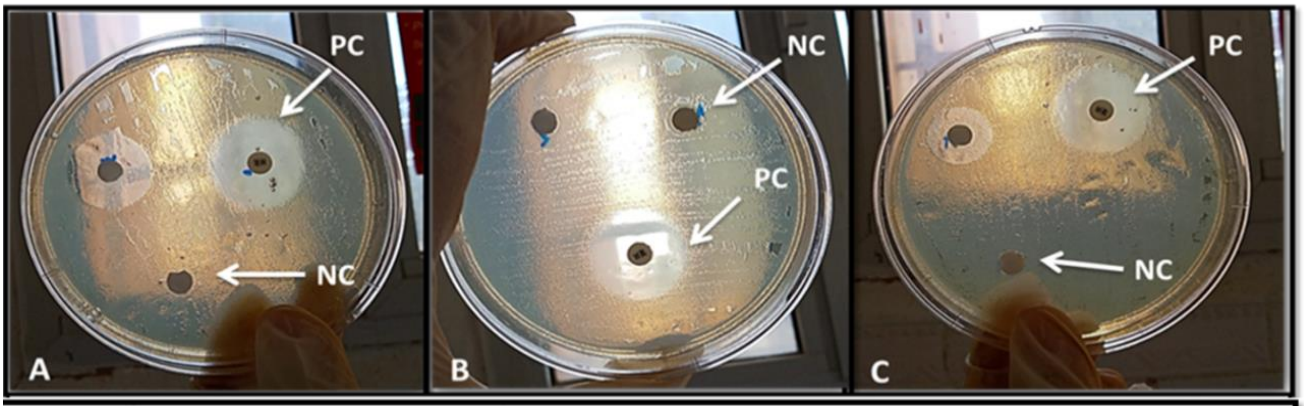

Figure 1. Antibacterial activity (inhibition zone) of *Allium sativum L.* and *Allium cepa L.* extracts against isolated *Escherichia coli* from broiler chickens affected by colibacillosis. **A**: *Allium sativum*; **B**: *Allium cepa*; **C**: *Allium sativum* + *Allium cepa*; PC: Positive control; NC: Negative control.

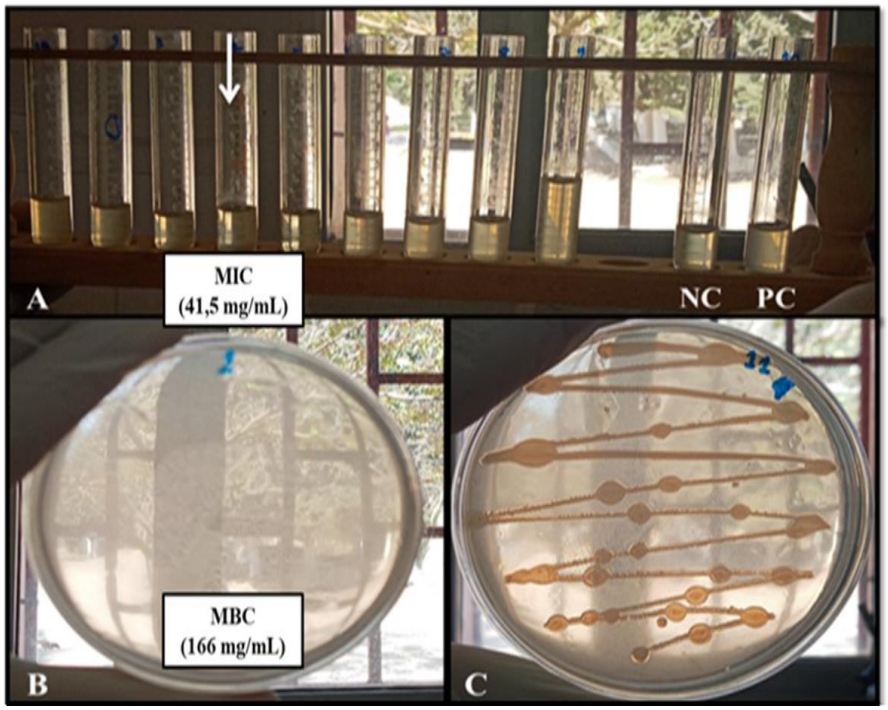

Figure 2. The minimal inhibitory concentration and minimal bactericidal concentration of *Allium sativum* extract against *Escherichia coli* isolated from broiler chickens affected by colibacillosis. **A**: MIC of *Allium sativum*; **B**: MBC of *Allium sativum*; **C**: Positive control for MBC.

DISCUSSION

The potential of *Allium sativum* L. and *Allium cepa* L. in combating multidrug-resistant bacteria has attracted considerable interest as an alternative therapeutic option. Previous studies have investigated the antibacterial effects of garlic and onion, particularly their efficacy against bacteria resistant to multiple drugs (Bhardwaj et al., 2016; Reiter et al., 2020). The current study aimed to assess the antibacterial properties of garlic and onion extracts against strains of *E. coli* that were resistant, and isolated from broiler chickens suffering from colibacillosis.

The obtained results revealed that *E. coli* resistant strains were extremely sensitive to the whole *Allium sativum* L. extract with an inhibition zone of 20.5 ± 1.29 mm, MIC of 41.5 mg/mL, and MBC of 166 mg/mL. These findings align partially with those of Magryś et al. (2021), who evaluated the antimicrobial activity of *Allium sativum* extract against various antibiotic-resistant bacterial strains. Their study demonstrated the extract's strong efficacy, particularly against multidrug-resistant *E. coli* and methicillin-resistant *Staphylococcus aureus*. Additionally, the *in-vitro* antibacterial activity of fresh garlic extract was evaluated against multidrug-resistant *E. coli* by Farrag et al. (2019), and reported that the MIC value was found to be 16 mg/ml. However, Noman et al. (2023) evaluated the antibacterial potential of garlic against different resistant bacteria isolated from chickens in Bangladesh and reported an inhibition zone of 14.03 ± 0.15 mm and a MIC of 0.625 mg/mL against *E. coli*. These variations could be due to the plant used or the bacterial strains used. Conditions related to the plant include genetic diversity, concentration of active compounds, climatic conditions, and farming techniques. Conditions related to bacterial strains include genetic background diversity, variations in drug resistance, and various methodologies used for isolation (Noman et al., 2023).

The positive antibacterial effect of garlic extracts on multidrug-resistant *E. coli* observed in the present study suggested that the antibiotic resistance mechanisms of *E. coli* do not influence its susceptibility to garlic. This was particularly attributed to its abundance of organosulfur compounds. The main organosulfur antibacterial compounds found in garlic were allicin, ajoenes, and various aliphatic sulfides (Bhatwalkar et al., 2021). Allicin was widely recognized as the most potent antibacterial agent found in crushed garlic extracts. Previous research has indicated that allicin effectively inhibits a wide range of infectious agents, including those that have developed resistance to conventional antibiotics (Bayan et al., 2014; Bhardwaj et al., 2016; Reiter et al., 2020). It offers a significant advantage over most antibiotics by not being limited to a specific protein within the bacterial cell. Consequently, the probability of resistance originating from alterations to the target site within the bacterial cell was reduced (Magryś et al., 2021).

According to the current study, a complete absence of the inhibition zone for *Allium cepa* L. extract was noticed, indicating a lack of antibacterial activity against the tested resistant *E. coli* strains. These findings align with those reported by Omotola et al. (2018), who tested onion aqueous extract against several pathogenic bacteria and found a total absence of antibacterial activity against *E. coli*, even at high concentrations. Furthermore, the same authors concluded that aqueous garlic extracts exhibited greater antimicrobial potential compared with onion extract. These researchers suggested that the reduced antibacterial activity of the onion extract could be attributed to the extraction method employed for the plant. This explains the results of the present study following the use of fresh onion juice.

Several studies have reported strong antibacterial activities of onion against numerous pathogenic bacteria using organic solvents for extraction. Bakht et al. (2013) reported that onion extracts in petroleum ether, ethyl acetate, and chloroform inhibited the growth of certain bacteria at both low and high concentrations. Eltaweel (2013) documented better antibacterial activity of methanolic onion extracts compared to aqueous extracts. Organic solvents were known to better dissolve organic compounds, thereby releasing the active component necessary for antimicrobial activity (Ekwenye and Elegalam, 2005).

CONCLUSION

The present study indicated that fresh *Allium sativum* juice exhibited a significant antibacterial effect on the resistant *E. coli* strains, with an inhibition zone diameter of 20.5 ± 1.29 mm, a MIC of 41.5 mg/mL, and an MBC of 166 mg/mL. Conversely, fresh *Allium cepa* juice was ineffective against the tested bacterial strain. Furthermore, the antibacterial effect of the combination of both extracts was inferior to that of garlic alone, with an inhibition zone diameter of 16.5 ± 0.57 mm. These findings suggested the potential use of garlic as an alternative to antibiotics in treating colibacillosis caused by a resistant strain of *E. coli*. It's essential to note that while the antimicrobial properties of *Allium sativum* showed promise, further research was needed to understand the specific mechanisms of action and to explore the potential development of garlic-based treatments or supplements for combating multidrug-resistant bacterial infections. Additionally, considering the dosage, formulation, and potential side effects should be thoroughly investigated for the practical application of *Allium sativum* in clinical aspects.

DECLARATIONS

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Authors' contributions
Rachid Merati contributed to the conception, design, data collection, analysis, interpretation, and writing. Abdellatif Boudra contributed to the data collection, analysis, editing, and writing the final draft of the manuscript. All authors approved the analyzed data and the last revised article.

Competing interests
The authors confirm that the data presented do not represent any conflict of interest.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethical considerations
Ethical issues (plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and submission, and redundancy) have been checked by all authors.

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