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In-vitro antimicrobial activity of *Lactuca Sativa* Leaves against Isolated Clarithromycin-resistant Superbugs

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Abstract

Antibiotics were one of the modern advancements in the 20th century, but they have been less active and have become more alarming due to antibiotic resistance. Antimicrobial resistance among pathogenic microorganisms is rapidly increasing, posing a danger to human health. However, the most essential biologically bioactive components are sourced by plants and are industrially used to produce drugs against several antibiotic-resistant bacteria. Antimicrobial agents based on plants possess fewer side effects and have immense potential than available drugs in clinics to combat superbugs. This study investigated bioactive components of Lactuca sativa (Lettuce) that were energetic in our research against Clarithromycin-resistant bacteria. *Lactuca sativa* had a substantially stronger antimicrobial effect on gram-negative bacteria than it did on gram-positive. Using a UV-visible spectrophotometer at 600nm, distinct behaviors of isolated bacteria were detected at varied optical densities; the highest activity was reported at 1 ml/50ml. Various phytochemicals were detected qualitatively, including carbohydrates, proteins, saponins, flavonoids, alkaloids, terpenoids, phenolic compounds, and tannins. Anthraquinones and glycosides were not discovered in lettuce. A quantitative investigation was conducted to detect unique phenolic compounds using High-Pressure Liquid Chromatography (HPLC) with varied peaks. Gallic acid, syringic acid, sinapic acid, and vanillin were identified as phenolic components by HPLC. However, further study on the analysis of isolated phytochemicals is required to identify novel antibiotics and their rapid and plant-based control and the proper management of antibiotic resistance spread and its risk to human health.

Keywords. *Lactuca sativa, lettuce, clarithromycin-resistance, bioactive components, phytochemicals*

INTRODUCTION

Antibiotic resistance refers to a microbe's capacity to thrive and suppress antibiotic activities, and it is a significant cause of a variety of illnesses and fatalities across the world (Kleina, E. Y., et al. 2018). In the past, infectious diseases were a major cause of death. However, developments in medicine and public health over the twentieth century helped reduce the burden of infectious diseases considerably. On the other hand, infectious diseases are on the rise once more, particularly those that can no longer be treated with previously available drugs. Infectious bacteria may acquire resistance to antibiotics, and many have developed resistance to both regularly used and newly developed medicines throughout time. On a global level, antibiotic resistance has become a huge threat to people's health. (Chokshi, A., & et al., 2019). Antibiotics were developed to give a simple and effective therapy for bacterial illnesses, and they have had a

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tremendous impact on human health and longevity since then. Antibiotic resistance (ABR) poses a concern to several organisms: Many pathogenic bacteria have developed resistance to the most common antibiotic classes, and multidrug-resistant bacteria have resulted in illnesses that are untreatable. ABR already has significant health and economic consequences, and by 2050, the worldwide yearly cost of ABR might reach 10 million fatalities and US$100 trillion (MacLean, R.C. and San Millan, A., 2019). Bacterial resistant strains (with immune genes) enter the water through faecal material, eventually distributing their genes (superbugs) to water-indigenous microorganisms (Baquero F. et al., 2008).

Antibiotic-resistant bacteria can be treated using a range of commercially available drugs. The fast growth of multidrug-resistant microorganisms, on the other hand, is jeopardising the therapeutic viability of many present medicines. Many known bacterial infections have been treated with natural items throughout human history (Naveed, R. et al., 2013). Bacterial resistant strains (with immune genes) enter the water through faecal material. Eventually, *Lactuca sativa* is a well-known universal plant because of its use in salads, soups, and vegetable curries. It also has excellent therapeutic effects. It is high in carotene, vitamin C, and vitamin E. Given the presence of these beneficial phytochemical constituents, it is assumed that the plant could have antioxidant potential (Hajare, A.G., et al., 2013). Despite this, there is a lot of room to explore plant potential to resist these human adversary superbugs, and the scientific community is seizing the issue of world health. In this study, it was tried to isolate Clarithromycin-resistant and -sensitive bacteria. To find the components present in *Lactuca sativa* active against those isolated bacteria.

LITERATURE REVIEW

Misuse and abuse of antibacterial medications, both in human health and agriculture, is one of the key driving causes behind the development of antibacterial drug resistance (Gajdács, M. and Albericio, F., 2019). Antibiotic resistance develops due to a variety of factors, including inappropriate antibiotic use, over-prescription, and patients who do not finish their antibiotic medication; antibiotic misuse in livestock and fish farming; poor infection control in healthcare settings; lack of hygiene and sanitation; unavailability of newly discovered antibiotics; and genetic mutations. Wastewater treatment accounts for just 1% of the industry in Pakistan, and sewage discharge without proper treatment is a major source of surface and groundwater contamination (Jabeen et al., 2015). Antimicrobial stewardship includes making judgments such as choosing the most appropriate antimicrobial(s) for the patient with the least amount of side effects, ensuring minimum influence on local resistance levels, and maintaining their availability and efficacy in the future (Dyar, O.J., & et al., 2017). Another growing component of antimicrobial stewardship is quick diagnostic procedures in clinical microbiology laboratories (diagnostic stewardship) to help in medication therapy selection (Morgan, D.J., Malani, P., and Diekema, D.J., 2017). Water habitats are relatively conducive for resistance gene transfer. Antibiotics and their resistance genes may be bypassing water treatment facilities, tipping the balances in favor of microorganisms gaining antibiotic resistance. Such resistance genes can be discovered in bacteriophages with wandering genetic sources, allowing for the unrestricted transmission of resistant features from one bacterium to another (Roopan, S.M. et al., 2016). A new antibiotic must be absorbed by body tissues in a consistent and timely manner. Antibiotics are most effective when it comes to destroying germs (Vineetha, N. et al., 2015). The efficiency of antibiotics in use throughout the world is gradually deteriorating - the medications we use are less effective. If the user does not take any action, the day will come when we will be unable to treat such bacterial infections with any medication. The scenario necessitates extensive research into novel, less costly, more secure, and dependable antimicrobial therapies. Natural ingredients are a tried-and-true option, and in recent decades, various plant research has been done to uncover novel anti-infection compounds (Sood, A. et al., 2012). According to WHO, almost 80% of the world population is entirely reliant on traditional medicine, which is blended for various ointments with plant chemical derivatives and raw extracts (Dar, P. et al., 2014). Traditional pants have made significant contributions to the advancement of modern medicine, particularly antimicrobials, which cure infectious disorders. To combat diseases, innovative antimicrobial agents with correct chemical structures and distinct mechanisms of action must be developed. The plant disinfectant has a broad restorative potential for a variety of infectious pathogens (Naveed, R. et al., 2013).

Hundreds of "superbugs" resistant to antibiotics have made headlines during the last quarter-century. Antimicrobial substances are also abundant in medicinal plants (Bebell, L. M., & Muiru, A. N. 2014). Traditional remedies are utilized by 80% of the people in Pakistan's rural areas (Javid et al., 2015). Although resistance has evolved to every antibiotic used in clinical practice, most efforts to treat AMR are focused on identifying new medicines. As a result, discovering new strategies to control the development of drug-resistant illnesses is a top concern for public health (Ragheb, M.N., & et al., 2019). Lettuce is one of the world's most commonly eaten leaf vegetables. Lettuce is abundant in water (95%) and fibers, as well as vitamins (A, B1, B2, B3, B9, C, and E), beta-carotene, phenolic compounds, minerals, and carotenoids. (Kim et al. 2016; Shams et al. 2019). Lettuce is one of the most often consumed raw edible plants and is high in phytonutrients, including vitamins, carotenoids, fiber, and phenols (Shahidi and Ambigaipalan 2015; Sönmez et al. 2017; Shams, M. et al., 2019). In vitro and in vivo studies have demonstrated that eating lettuce offers health benefits, including decreasing cholesterol (Lee et al. 2009), inflammatory activities (Pepe et al. 2015), diabetes (Cheng et al. 2014), and other disorders (Pelegrino, M.T., & et. Al., 2020). Non-nutritive bioactive plant compounds with disease-fighting or prevention properties are known as phytochemicals. These bioactive components are responsible for plant extracts' antibacterial activity in vitro (Bhat, R.S. and Al-Daihan, S., 2014).

RESEARCH METHODOLOGY

Isolation and Characterization of Isolated bacteria:

Clarithromycin-resistant and -sensitive microorganisms (*Staphylococcus aureus, Escherichia coli, Salmonella typhi, and Shigella flexneri*) were isolated. They characterized using culture and biochemical identification of microorganisms from samples taken from tap water.

Sample preparation:

Lactuca sativa was bought from a local market in Latifabad, Sindh, and cleaned with tap water before being cleansed with distilled water. One gram of plant yielded 200-300 µl of crude extract. Clarithromycin and plant extract were each diluted in distilled water separately. The test substances were then put onto the 6mm antibiogram plane discs.

Susceptibility Testing:

To test the susceptibility of *Lactuca sativa*, a disc diffusion technique was used. Antibiotic-loaded discs served as the control group, whereas *Lactuca sativa* extract-loaded discs served as the treatment group. The measurements were obtained in terms of zones of growth inhibitions. The turbidity levels were used to determine the MIC of extract for all bacterial strains, which had been quantified with a UV-Visible Spectrophotometer at 600nm.

Hight-throughput Phytochemical Screening:

High-throughput phytochemical screening of extract was conducted to detect carbohydrates, amino acids, saponins, phenolic compounds, tannins, and proteins, qualitatively (Santhi, K. and Sengottuvel, R., 2016; Bhandary, S.K. et al., 2012). Anthraquinones, flavonoids, glycosides, alkaloids, and terpenoids were also discovered (Krishnapriya, T.V., and Suganthi, A., 2017; Hajare, A.G.,2013; Santhi, K. and Sengottuvel, R., 2016; Doss, A. 2009).

Quantitative analysis of Phenolic Compounds through HPLC:

The quantitative analysis of lettuce extract was performed at the National Centre of Excellence in Analytical Chemistry. Free and bound phenolic acids from *Lactuca sativa* extract was separated using Spectra System SCM 1000 (Thermo Finnigan, California, USA) liquid chromatograph equipped with a diode array detector system. U.V. analysis was performed at 270, 310, and 325 nm using the DAD package. Individual phenolic acid structures were validated by relating their U.V. spectra and retention time (tR) to the applicable parameters. Based on the peak area of their associated standard curves, the concentrations of the chemicals were figured (Siddiqui, M.S. et al., 2017).

FINDINGS AND DISCUSSION

Antibacterial Screening of *Lactuca Sativa* Extract

As stated in table 1, a crude extract of *Lactuca sativa* at a concentration of 10µl/50µl demonstrated the lower and higher efficiency against clarithromycin-resistant (R25) *S.flexneri* (10.6±0.6mm) and (R100) *S.typhi* (11.3±3.3mm), respectively.

Table 1. Antibacterial Screening of *Lactuca sativa* against Clarithromycin-resistant and -sensitive pathogens

Microbial strain	***Concentration with Zone of inhibion*** **((Mean±SE))** ***of Lactuca sativa*** **Extract**			
	5µl	**10µl**	**20µl**	**30µl**
E.coli **(S)[1]**	0	10.1±1.1	12.0±2.3	12.61±1.7
E.coli **(R50)[2]**	0	0	17.31±0.6	19.32±0.6
S. typhi **(S)**	8.61±0.6	9.32±0.6	11.33±0.6	14.21±0.6
S. typhi **(R100)[3]**	8.62±0.6	11.31±3.3	13.32±2.4	14.61±1.7
S. flexneri **(S)**	8.60±0.6	9.30±1.3	10.1±1.1	12.21±2
S. flexneri **(R25)[4]**	9.34±0.6	10.63±0.6	12.61±0.6	14.21±2
S. aureus **(S)**	8.1±0	8.61±0.6	10.11±0	10.63±1.7
S. aureus **(R25)**	0	0	10.12±0	10.61±0.6

[1](S)= Sensitive to Clarithromycin, [2]R (50) = Resistant at 50 µl, [3](R100)= Resistant at 100 µl, [4](R25)=Resistant at 25 µl (p-value < 0.5)

The crude extract concentration of 20µl/50µl revealed that (R25) S.flexneri (12.6±0.6mm) had the lowest antibacterial activity, whereas (R50) E.coli (17.3±0.6mm) and (R25) S.aureus (10 ±0mm) had the highest antibacterial activity. The significant efficacy of *Lactuca sativa* extract was reported against resistant bacterial strains at a concentration of 30µl/50µl.

MIC of *Lactuca sativa* Extract against Resistant Microbes

To estimate the MIC of *Lactuca sativa* extract, optical density was measured at 600nm. Various growth inhibition behaviours were detected in the presence of extract. The maximum MIC was reported against gram-negative bacteria, as illustrated in figures 1A, B, C, and D. *Lactuca sativa* had the highest MIC at 500µl/ml against clarithromycin (S) *E.coli* at 0 and 24 hours. While testing against (R50) *E.coli*, good MIC was obtained at the concentration of 500µl/ml at 0 hours, as shown in figure 1 A. *Lactuca sativa* extract had the greatest MIC against

(S) S.typhi at 500lµl/ml at 0 hours, excellent activity at 125µl/ml, and the best MIC activity at 500µl/ml during 24 hours. Similarly, for resistant S.typhi, the MIC was 125µl/ml at 0 hours and 125 µl /ml after24 hours, as illustrated in figure 1 B. At 0 hours, Lactuca sativa extract had the greatest MIC against (S) S. flexneri at a dosage of 250µl/ml. For (R25) S. flexneri, the extract had the highest activity at a concentration of 250µl/ml at 0 hours. It had the best MIC at 24 hours at 125µl/ml, as illustrated infigure 1C. *Lactuca sativa* extract had a good MIC at a concentration of 250µl/ml against (S25) *S. aureus* at 0 hours and 125µl/ml after 24 hours, indicating inhibition of bacterial growth. As demonstrated in figure 1D, the most significant MIC against (R) *S.aureus* was observed at 500µl/ml at 0 h and 125l/ml at 24 hours.

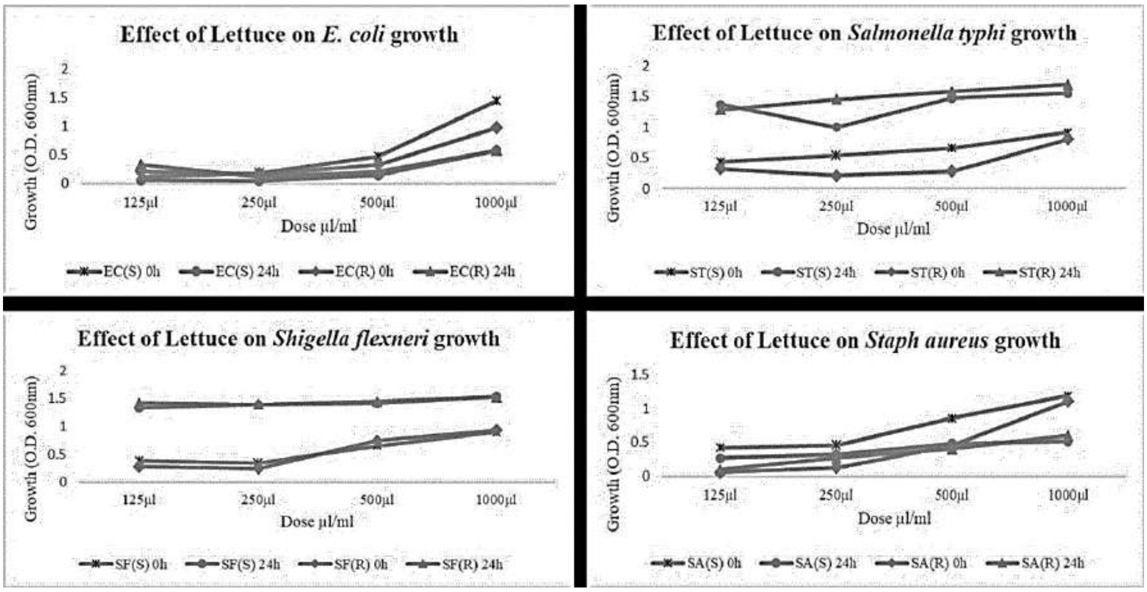

Figure 1. Effect of different concentrations of Lettuce aqueous extract on isolated resistant and sensitive microbes: A) effect on the growth of *E.coli*, B) effect on the growth of *Salmonella typhi*, C) effect on the growth of *Shigella flexneri*, and D) effect on the growth of *Staph aureus*

Qualitative Phytochemical Analysis of *Lactuca Sativa* Extract

The phytochemical contents of *Lactuca Sativa* were studied qualitatively. *Lactuca sativa* extract included carbohydrates, proteins, saponins, flavonoids, alkaloids, terpenoids, phenolic chemicals, and tannins. Anthraquinones and glycosides, on the other hand, were not found in *Lactuca Sativa* extract.

HPLC of *Lactuca Sativa*

The phenolic compounds present in *Lactuca Sativa* that may be accountable as bioactive components in *Lactuca Sativa* (Lettuce) extract against the Clarithromycin-resistant bacteria were quantified using High-Pressure Liquid Chromatography (HPLC).

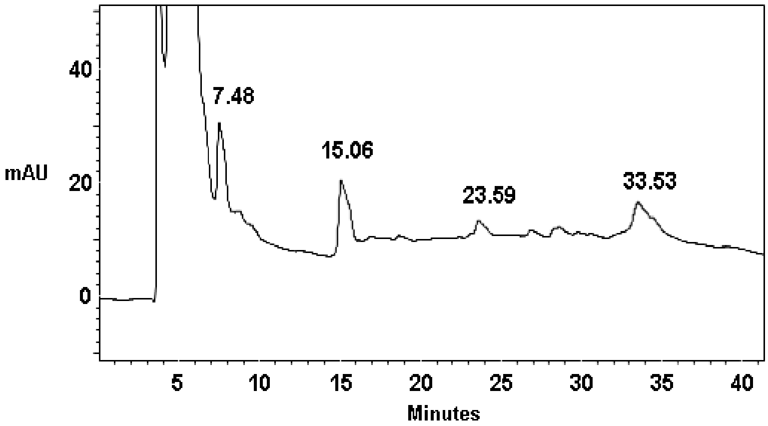

Figure 2. HPLC separated fractions from Lettuce water extract.

Figure 2 depicted four distinct phenolic compounds as bioactive components at varying retention times and in varying amounts. Gallic acid was discovered at 42.1677 µg/ml at a retention time of 7.48, while syringic acid was detected at 49.7982 µg/ml at a retention time of 15.06. Sinapic acid was 12.4573µg/ml after 23.59 retention periods. Vanillin had a retention time of 33.53 and a concentration of about 68.0183µg/ml.

CONCLUSION

In this study, Clarithromycin-resistant bacteria were isolated and identified form drinking water; the *E.coli* colonies were found resistant at 50µg/mL, *S.typhi* at 100µg/mL, and *S.flexneri* and *S.aureus* at 25µg/mL of extract concentration. Microbial inhibitory activity against resistant *E.coli* was the highest in *Lactuca Sativa* (Lettuce), whereas it was the lowest against sensitive and resistant *S.aureus*. The extract of *Lactuca Sativa* included alkaloids, phenolic compounds, tannins, and sapssssonins, all of which are important antibacterial ingredients and carbohydrates and proteins, which are required macromolecules with nutritional value. The phenolic compound detected in *Lactuca sativa* (Lettuce) were gallic acid, syringic acid, sinapic acid, and vanillin. Further study by comparing with other vegetable extracts should be carried out to develop natural-based drugs to combat such superbugs.

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