

IN VITRO EVALUATION OF THE ANTIBACTERIAL POTENTIAL OF TRACHYSPERMUM AMMI (AJWAIN) ETHANOLIC EXTRACT AGAINST CLINICAL ACINETOBACTER BAUMANNII ISOLATES

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Keywords

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Abstract

Background: Acinetobacter baumannii is a highly resistant Gram-negative pathogen with the decreasing availability of effective antimicrobial agents, which has raised interest in naturally occurring and plant-derived antimicrobial compounds. Trachyspermum ammi (ajwain) is a medicinal aromatic spice with bioactive compounds like thymol and carvacrol, that have shown antimicrobial properties.

Objective: To assess the in vitro antibacterial activity of the ethanolic extract of T. ammi against clinical isolates of A. baumannii by the agar well diffusion and broth microdilution methods.

Materials and Methods: Ajwain seed was cleaned, dried, powdered, and extracted with ethanol. The initial screening for the antibacterial activity was done with the agar well diffusion method against standardized bacterial suspensions (0.5 McFarland) on Mueller–Hinton agar. Colistin was used as a positive control and dimethyl sulfoxide (DMSO) was used as a negative control. The minimum inhibitory concentration (MIC) was then calculated by two-fold serial dilutions of the extract in 96-well microtiter plate. Optical density was measured at 600 nm, prior to and after 24 h incubation. Mixed ANOVA was used to evaluate the effect of the extracts and bacterial strains.

Results: Ethanolic extracts were found to have measurable inhibitory effect against all three A. baumannii isolates with the inhibition zone being 15mm, 14mm and 13mm, respectively (mean inhibition zone = 14.0 ± 1.0 mm). The MIC values were 50 mg/mL for isolates 2 and 3, and 50 and 100 mg/mL for isolate 1 in two determinations. The concentration of extract had significant impact on the bacterial growth ($p < 0.001$) and significant difference between the strains ($p = 0.048$).

Conclusion: Ethanolic T. ammi extract demonstrated in vitro antibacterial activity against A. baumannii and should be investigated, employing standardized extraction methods, phytochemical characterization, and expanded collections of A. baumannii.

1. INTRODUCTION

Acinetobacter baumannii is a Gram-negative bacterium that is widely prevalent and has also been linked to severe infections in both hospitals and communities. It is a severe health issue of the population because it has become resistant to the last-resort antibiotics such as Colistin, Tigecycline, and Carbapenems. Its ability to form biofilms, to endure in adverse environments, and its resistance to disinfectants enable it to grow in clinical settings. Although there are no apparent toxins, it escapes the immune system through a so-called persist and resist strategies like enzyme degradations and biofilm formation. *Acinetobacter baumannii* is an increasing and severe problem in a clinical environment (Vázquez-López et al., 2020). Colistin is one of the final antibiotics that is used for the treatment of multidrug-resistant Gram-negative bacterial infections and is predominantly used to treat *A. baumannii* infections. Nevertheless, the bacteria with their resistance factors seriously restrict the therapeutic opportunities and are linked to the rise of morbidity and mortality (Islam, Mahbub, et al., 2024).

This resistance is due to the ability of the bacterium to persist over a long duration of time in the harsh environment of hospitals, and hence, it can spread in health care facilities. *Acinetobacter baumannii* primarily targets people who also have illnesses or are sick, especially those who have compromised skin barriers, or those who are immunocompromised or those with impaired respiratory immunity. While hospital-acquired pneumonia has been recognised as the most common *A. baumannii* infection, recent reports highlight the rising prevalence of infections affecting the central nervous system, skin and soft tissues, and bones, posing significant challenges in certain healthcare institutions (Shi et al., 2024a). The rise of antibiotic resistance due to overuse of the available antibiotics and failure to develop new antibiotics, that has created a pressing demand for other antimicrobial modalities (Whiteway et al., 2022). The increases in the rate of *A. baumannii* infections indicate the urgent need for alternative antibacterial agents that could be natural in their origin, like *Trachyspermum ammi* (ajwain). Plants

contain a high number of chemical compounds that are used in medicine. There is a growing popularity of natural therapy for bacterial diseases. Many modern medicines are derived from plants (Ehyaeirad et al., 2024). Many research teams across the globe have reinstated their interest in uncovering the advantages of plant extracts and taking full advantage of them as acceptable alternatives to antibiotics. The extraction is performed on plant active components using different organic and inorganic solvents, and then, it is purified by chromatography. Herbs enhance the response of the host to the immunization, and it leads to the enhancement of infection prevention and control. Virgin coconut oil, *Echinacea purpurea*, *Myrtus communis* oil, *Allium Sativum* powder, *Trachyspermum ammi* (ajwain), and turmeric is quite helpful in the given case. In recent research, the researcher aims to investigate their impact by conducting in vitro experiments of their effect on bacterial growth, and also provides a step-by-step instruction to the scientists and workers in the field on how to develop new and effective herbal-based drugs to prevent and inhibit bacterial growth.

Trachyspermum ammi (Ajwain) is a spice that is employed in traditional medicine to treat a variety of conditions because it has a wide range of therapeutic benefits. Its key active constituents are thymol and carvacrol, which are attributed to its intense aroma and biological activity. Thymol is the most important bioactive ingredient of these that has been known to have strong antimicrobial and antioxidant properties. *Trachyspermum ammi* (Ajwain) has been proven to have antibacterial action against several pathogenic bacteria, such as *A. baumannii*, *Escherichia coli*, *Staphylococcus aureus*, as well as *Salmonella typhi* (Mayekar et al., 2021). Thymol and carvacrol affect the cell membranes of the bacteria by causing leakage of cell contents and stopping some essential metabolic activities. This is the mechanism that renders ajwain a good natural antimicrobial agent that could find use in food preservation, pharmaceuticals, and alternative medicine (Shiva et al., 2024).

The purpose of the present investigation was therefore to determine whether an ethanolic

extract of *T. ammi* could inhibit growth of clinical *A. baumannii* isolates under controlled in vitro conditions. Unlike studies evaluating multiple formulations, this work concentrates on the antibacterial performance of the ajwain extract itself, providing a focused assessment of its activity against five clinical isolates.

2. MATERIAL AND MATHODS

2.1. Sample collection

Three isolates of *Acinetobacter baumannii* isolates were procured from Mughal lab, Lahore. And testing substance *T. ammi* (Ajwain) collected from local market of Lahore.

2.2. Media Preparation

All of the media (MacConkey and Muller Hinton Agar) and reagents required were prepared in accordance with manufacturer's instructions. The pH of media was adjusted according to manufacturer's instructions by using NaOH and HCl. The sterility of each medium was checked after 24 hours aerobic incubation at 37°C.

2.3. Bacterial Identification

The clinical isolates of *Acinetobacter baumannii* were first grown on MacConkey agar to isolate and purify. Bacterial growth was followed after incubation for the characteristics of the colonies and colonies that appeared pink to off-white were picked and sub cultured to get pure cultures. Then, purified colonies were stained with Gram stain and observed with light microscope to identify their Gram reaction and the morphological characteristics of their cells. Isolates with the characteristics were tested for further confirmation of *Acinetobacter* using appropriate biochemical tests that included short coccobacillary cells and the Gram-negative character of the cells (Shi et al., 2024b). After identification, confirmed isolates of *A. baumannii* were tested for antibiotic susceptibility testing (AST) to check for susceptibility or resistance to the selected antibiotics including colistin.



A. 3 isolated of *Acinetobacter baumannii*

Figure 2.1 Isolates of *Acinetobacter baumannii*

2.4. Preparation of Ajwain Extract

Ajwain (*T. ammi*) seeds which are capable of generating strong antimicrobial and antifungal effects because of the presence of such bioactive substances as thymol, carvacrol and terpenes. The seeds were adequately wiped to get rid of dust and contaminants. Dried they were ground in a sterilized mortar and pestle to a rough powder. About 10g of ajwain powder was soaked with 100mg of ethanol and left to sit for 24 to 48 hours at the room temperature or at controlled conditions (Panda et al., 2020) . After the extraction process was done, it was filtered using Whatman filter paper in order to get a clear extract devoid of solid residue and afterwards put in an incubator to obtain a semi-liquid extract.

2.5. Anti-microbial activity of Ajwain by Agar Well Diffusion Assay

The antibacterial activity of ajwain extract against the 5 isolates of *Acinetobacter baumannii* was studied by agar well-diffusion technique. A new culture was started for each isolate of *A. baumannii* and the bacterial suspension was then standardized to approximately 0.5 McFarland standard. An even spread of the standardized inoculum was done over the surface of Mueller-Hinton agar (MHA) plates by means of a sterile swab in order to obtain a uniform bacterial lawn (Zhu et al., 2019) . The inoculated agar was then used to make sterile wells. Test wells were filled with Ajwain extract in diluent of dimethyl sulfoxide (DMSO) at 100 mg/mL. As the positive control, colistin was added to separate wells, and DMSO alone was added as the negative control. This was carried out individually for each of the five bacterial isolates. The inoculated plates were then incubated at 37°C for 18-24 hours to give rise to bacterial growth and diffusion of the compounds tested into the agar plate. Plates were then observed for clear zones that surrounded the

wells after incubation. The zone of inhibition diameter of the ajwain extract and colistin were measured in millimetre (mm) and recorded. The DMSO control was tested to ensure that DMSO itself did not inhibit bacterial growth. A clear zone around the ajwain extract indicated inhibition of the growth of *A. baumannii* and comparison with the positive and negative control helped to assess whether the antibacterial activity was due to ajwain extract or not.

2.6. Minimum inhibitory concentration

Broth microdilution method can be used for the determination of minimum inhibitory concentration (MIC) with serial two-fold dilutions of the ethanolic extract of the plant and a standardized inoculum of the bacteria. The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic that will inhibit bacterial growth when tested under specific conditions.

2.7. Data Analysis

The inhibition zone data was summarized descriptively. The mean and standard deviation of the reported measurements were computed. Data were analysed using SPSS software. The data were analysed using mixed ANOVA.

3. RESULTS

3.1. Zone of Inhibition

The *T. ammi* ethanolic extract showed measurable response to inhibit the growth of all three tested *A. baumannii* isolates. The inhibition zones were between 13 and 15 mm wide, with isolate 1 producing 15 mm, 14 mm for isolate 2 and 13 mm for isolate 3 which was the largest observed. Hence, the difference in the minimum and maximum recorded inhibition zones was only 2 mm.

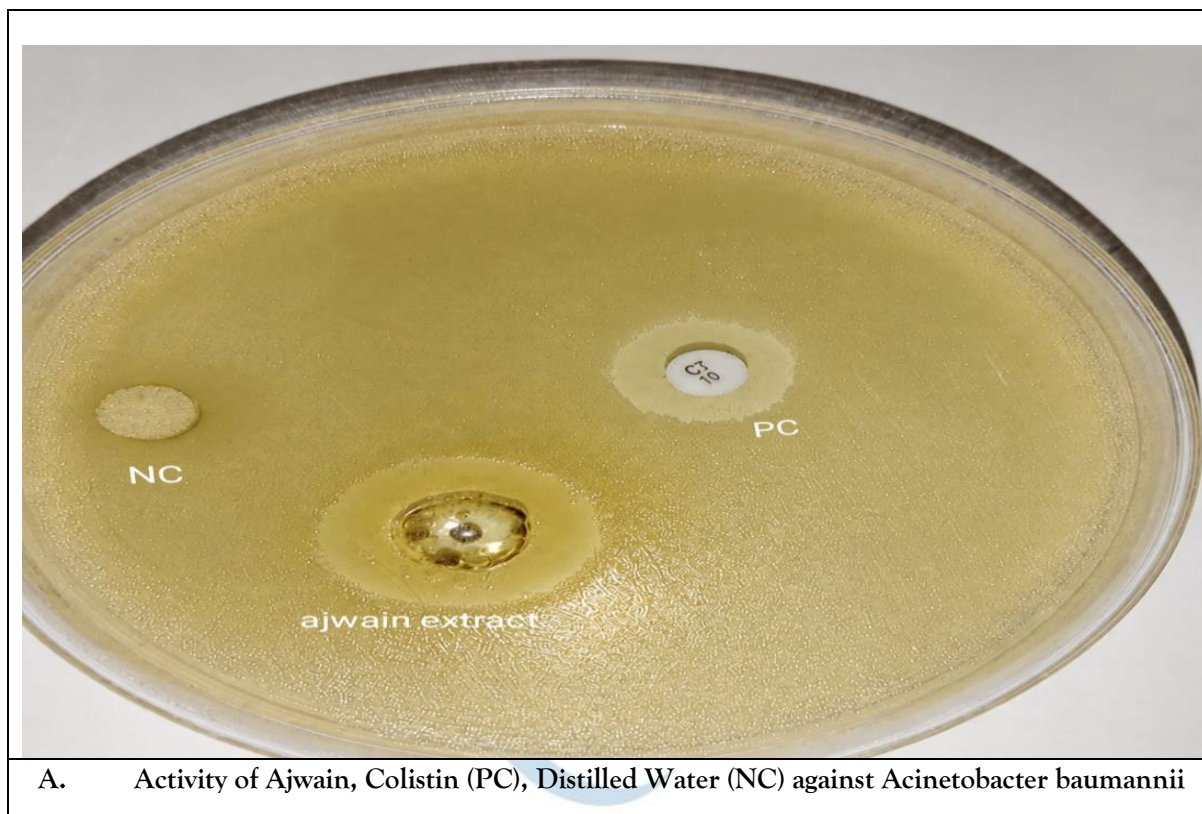


Figure 3.1 Activity of Ajwain (*T. ammi*), Colistin (PC), Distilled Water (NC) against *Acinetobacter baumannii*

Table 3.1 Zone of Inhibition of Ajwain (*T. ammi*), Colistin (PC), Distilled Water (NC) against three isolates of *Acinetobacter baumannii*

Isolate	<i>T. ammi</i> inhibition zone (mm)	Observation
Isolate 1	15	Inhibition observed
Isolate 2	14	Inhibition observed
Isolate 3	13	Inhibition observed
PC (colistin)	13	Inhibition observed
NC (distilled water)	0	No inhibition observed
Mean $\pm$ SD	14 $\pm$ 0.84	Descriptive summary

3.2. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of *Acinetobacter baumannii* against ajwain ethanolic extract was determined by broth microdilution method using 96 well micro-titer plate (Kowalska-Krochmal & Dudek-Wicher, 2021). The ajwain ethanolic extract was serially diluted two times and bacterial suspension was added to all the wells of the plate. The extract with the lowest concentration from the graph that showed

inhibition of visible growth of the bacteria was determined as shown in Figure 4.14. After 24 hours, the optical density (O.D.) of the solutions was measured and MIC values were calculated by comparing the result with the preincubation value. O.D. values were measured at 600nm, both at 0 hours and after incubation for 24 hours, see Figures 4.12 and 4.13 respectively. In all cases, strain 2 and strain 3 exhibited an MIC at 50 mg/ml while strain 1 exhibited the MIC at 50

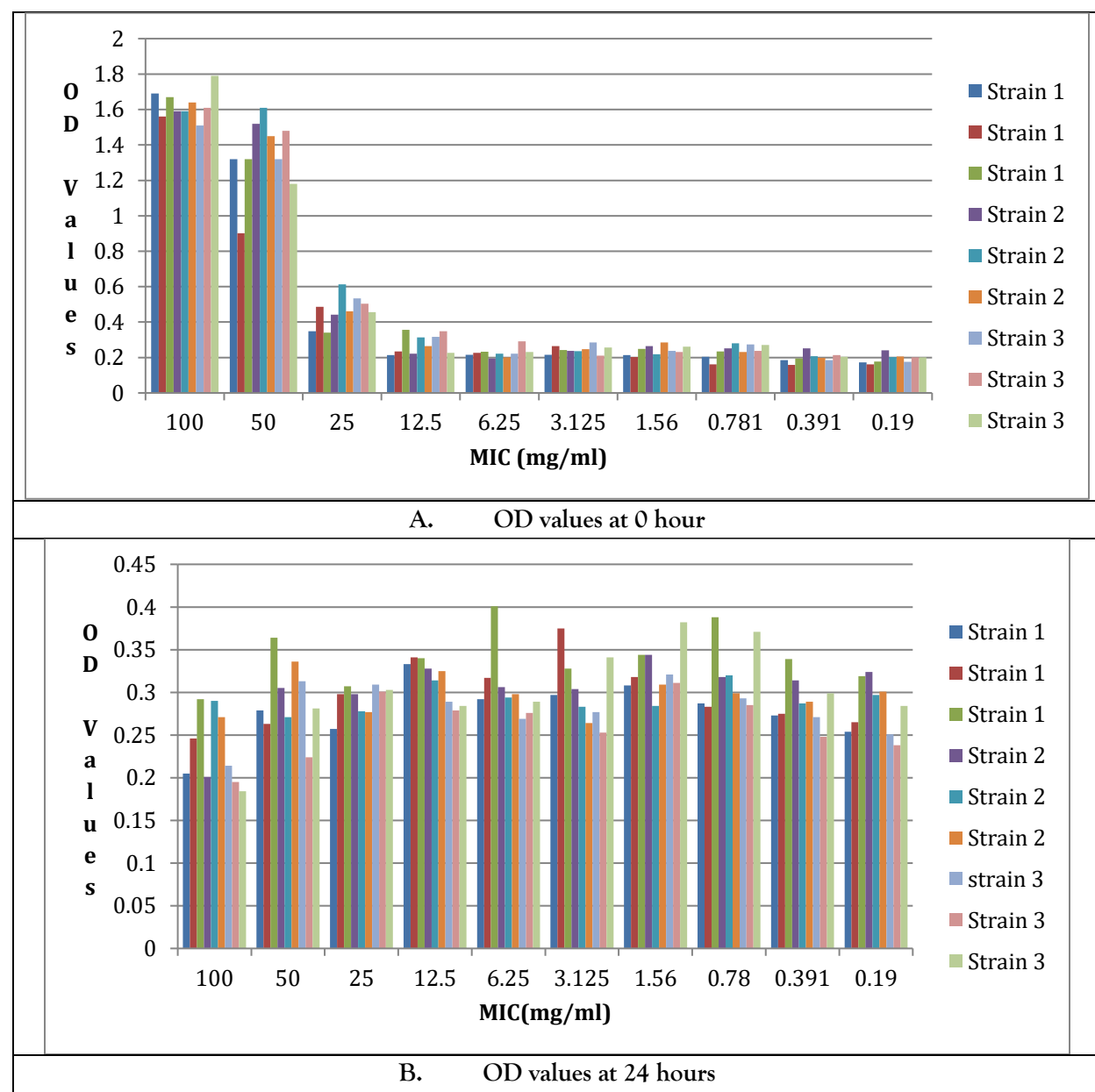
mg/ml and 100 mg/ml. Finally, statistical analysis was used to analyse the results. Mixed ANOVA was used to test the effect of varying concentration of ajwain ethanolic extract and the variation among different strains. The statistical analysis of within-subject effects, (concentrations) showed significant differences between the average effects of the concentrations ($p = 0.000$). The between-subject effects (strains) also showed significant differences between the average effects of the strains ($p = 0.048$) suggesting that at least two strains exhibited significantly different response to the extract. The results showed that ethanolic extract of ajwain (50mg/ml) was effective enough

to inhibit growth of most of the *A. baumannii* strains tested in the current study, but strain dependent activity was seen at this concentration with 100mg/ml required to inhibit growth for strain 1. The agreement of the MICs obtained from the replicates and the responses of appropriate controls indicate the reliability of the observations made. In general, the results show that ajwain ethanolic extract has antibacterial activity against *A. baumannii* and suggests the need to take specific strains into account when assessing its inhibitory properties.

	100mg/ml	50	25	12.5	6.25	3.125	1.56	0.781	0.391	0.19	PC	NC
Strain 1	1.69	1.32	0.348	0.213	0.216	0.215	0.213	0.205	0.185	0.172	0.161	0.157
Strain 1	1.56	0.901	0.486	0.234	0.226	0.264	0.202	0.161	0.158	0.161	0.161	0.155
Strain 1	1.67	1.32	0.341	0.356	0.232	0.243	0.249	0.234	0.194	0.178	0.157	0.155
Strain 2	1.59	1.52	0.442	0.221	0.195	0.237	0.265	0.252	0.251	0.24	0.152	0.156
Strain 2	1.59	1.61	0.613	0.314	0.221	0.236	0.218	0.281	0.208	0.202	0.155	0.158
Strain 2	1.64	1.45	0.461	0.264	0.201	0.247	0.285	0.231	0.198	0.206	0.161	0.156
Strain 3	1.51	1.32	0.534	0.317	0.221	0.285	0.237	0.274	0.185	0.175	0.153	0.157
Strain 3	1.61	1.48	0.503	0.348	0.292	0.211	0.231	0.238	0.214	0.201	0.144	0.164
Strain 3	1.79	1.18	0.456	0.227	0.231	0.256	0.262	0.271	0.204	0.201	0.159	0.154
A. OD values at 0 hour												
	after 24 h 100mg/ml	OD Final 50	25	12.5	6.25	3.125	1.56	0.781	0.391	0.19	PC	NC
Strain 1	0.205	0.279	0.257	0.333	0.292	0.297	0.308	0.287	0.273	0.254	1.23	0.272
Strain 1	0.246	0.263	0.298	0.341	0.317	0.375	0.318	0.283	0.275	0.265	1.19	0.151
Strain 1	0.292	0.364	0.307	0.34	0.401	0.328	0.344	0.388	0.339	0.319	1.22	0.152
Strain 2	0.201	0.305	0.298	0.328	0.306	0.304	0.344	0.318	0.314	0.324	0.857	0.152
Strain 2	0.29	0.271	0.278	0.314	0.294	0.283	0.284	0.32	0.287	0.297	0.968	0.153
Strain 2	0.271	0.336	0.277	0.325	0.298	0.264	0.309	0.299	0.289	0.301	0.873	0.152
Strain 3	0.214	0.313	0.309	0.289	0.269	0.277	0.321	0.293	0.271	0.251	0.941	0.155
Strain 3	0.195	0.224	0.301	0.279	0.276	0.253	0.311	0.285	0.248	0.238	1.01	0.166
Strain 3	0.184	0.281	0.303	0.284	0.289	0.341	0.382	0.371	0.299	0.284	1.09	0.159
B. OD values after 24 hours												
	100mg/ml	50	25	12.5	6.25	3.125	1.56	0.781	0.391	0.19	PC	NC
Strain 1	-1.485	-1.041	-0.091	0.12	0.076	0.082	0.095	0.082	0.088	0.082	1.069	0.115
Strain 1	-1.314	-0.638	-0.188	0.107	0.091	0.111	0.116	0.122	0.117	0.104	1.029	-0.004
Strain 1	-1.378	-0.956	-0.034	-0.016	0.169	0.085	0.095	0.154	0.145	0.141	1.063	-0.003
Strain 2	-1.389	-1.215	-0.144	0.107	0.111	0.067	0.079	0.066	0.063	0.084	0.705	-0.004
Strain 2	-1.3	-1.339	-0.335	0	0.073	0.047	0.066	0.039	0.079	0.095	0.813	-0.005
Strain 2	-1.369	-1.114	-0.184	0.061	0.097	0.017	0.024	0.068	0.091	0.095	0.712	-0.004
Strain 3	-1.296	-1.007	-0.225	-0.028	0.048	-0.008	0.084	0.019	0.086	0.076	0.788	-0.002
Strain 3	-1.415	-1.256	-0.202	-0.069	-0.016	0.042	0.08	0.047	0.034	0.037	0.866	0.002
Strain 3	-1.606	-0.899	-0.153	0.057	0.058	0.085	0.12	0.1	0.095	0.083	0.931	0.005
C. OD differences (24h-0h)												

	100mg/ml	50	25	12.5	6.25	3.125	1.56	0.781	0.391	0.19	PC	NC
Strain 1	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 1	neg [MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 1	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 2	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 2	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 2	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 3	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 3	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg
Strain 3	neg	neg[MIC]	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg

D. Interpretation of MIC

Figure 3.2 Optical Density Values of Ajwain (*T. ammi*) at 600nm

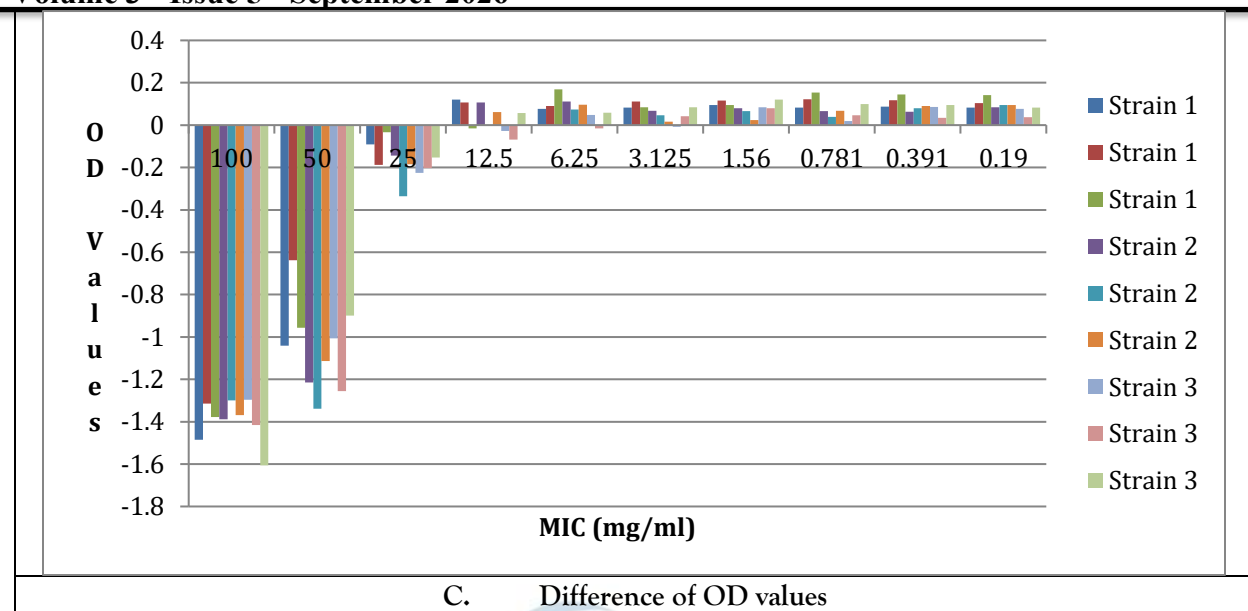


Figure 3.3 Graphical Representations of Optical Density Values

4. DISCUSSION

The emergence of AMR in *A. baumannii* has been growing a demand for antimicrobial sources other than antimicrobials, especially those of medicinal and aromatic plants. The plant extracts are rich in various bioactive substances that can have multiple mechanisms of action and thus serve as an important research focus for the exploration of complementary antimicrobial strategies (Islam, Jung, et al., 2024). The aromatic medicinal plant Ajwain (*Trachyspermum ammi*) is used for its medicinal properties and is known to possess various biologically active constituents such as components of essential oil and phenolic compounds. The present study offers an opportunity to recover a wide range of polar and moderately non-polar phytochemicals which could be species of antibacterial activity using the ethanolic extraction method. Plant variety, geographical origin, extraction solvent used, extraction conditions, phytochemical composition and type of microorganism targeted are some of the factors that can affect the antibacterial activity of plant extracts (Modareskia et al., 2022).

Furthermore, the structure of bacterial cells, the permeability of bacterial membranes, bacterial efflux activity, bacterial biofilm production, and/or intrinsic resistance or acquired resistance

mechanisms could contribute to the variation in the response of *A. baumannii* to plant compounds (Müller et al., 2023). Such factors are important in the investigation of multidrug resistant organisms, where traditional antimicrobial therapy may be difficult. The antimicrobial activity of ajwain could be attributed to the phenolic constituents of ajwain which interact with the bacterial membranes, affect the permeability of membranes, disturb cellular activities and interfere with the essential metabolic activities (Mueed et al., 2023). Multiple phytochemicals may also have additive and/or synergistic effects. But, the antibacterial activity of a crude plant extract cannot be explained by any single compound without proper phytochemical and mechanistic studies.

4.1. Conclusion

The present study validates the study of ajwain (*Trachyspermum ammi*) ethanolic extract for its antibacterial potential against *Acinetobacter baumannii*, a source from plant world. The study lays a foundation for future research on the phytochemical components that are responsible for its biological activity. Plant extracts are complex mixtures of compounds and detailed chemical characterisation and mechanistic

investigations are needed to identify those compounds which contribute to antibacterial effects. Purified compounds should be further studied for their activity, any interactions with known antibiotics, and safety. These investigations might contribute to the understanding of the possible potential of ajwain compounds in future antimicrobial research and development.

4.2. Limitations

The limitations of the study include the use of crude ajwain ethanolic extract and few strains of *Acinetobacter baumannii* were used in the study. No information on the active compounds nor their mode of action against bacteria. In addition, only in-vitro testing was performed, and toxicity, stability, and clinical effectiveness were not evaluated. Larger number of isolates should be subjected to further studies and phytochemical analysis and in vivo studies are recommended.

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