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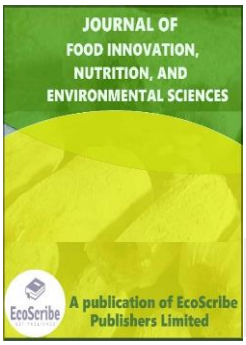

ORIGINAL ARTICLE

Efficacy Evaluation of *Jania rubens* Extract Against Four Pathogenic Strains Associated with Foodborne Disease in Iran

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ABSTRACT

This study evaluated the antimicrobial properties of *Jania rubens* algae extract, obtained via Soxhlet extraction, against food spoilage and pathogenic bacteria. Using response surface methodology and the Box-Behnken design, the research optimized extraction parameters, including solvent type, solvent-to-algae ratio, and extraction duration. Antimicrobial efficacy was assessed through the zone of inhibition (ZOI), Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) against *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Bacillus cereus*. Results showed MIC and MBC values for *E. coli* ranged from 0.32–1.5 and 2–3, respectively; for *S. enteritidis*, 0.98–1.9 and 2.5–3.5; for *S. aureus*, 0.4–1.5 and 0.8–2.8; and for *B. cereus*, 0.36–1.35 and 0.85–1.85. Optimal extraction conditions included acetone as the solvent, a 5:1 solvent-to-algae ratio, and extraction durations of 2 or 6 hours. The algae extract significantly extended the lag phase and reduced bacterial growth rates, with effects intensifying at higher concentrations. The study concludes that *Jania rubens* extract, particularly under optimized Soxhlet extraction conditions, exhibits potent antibacterial activity, making it a promising natural antimicrobial agent for food preservation.

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1. Introduction

Ensuring the safety and improving the quality of food during its storage is an urgent concern that captures the interest of experts in the food industry and health officials across various countries. Failure to address this issue can result in severe and lasting consequences for society. The incidence of outbreaks arising from the intake of contaminated food is recognized as a major global issue, impacting even those developed nations and countries with advanced economies; Globally, it is estimated that approximately 600 million individuals, which equates to nearly 10% of the population, suffer from illnesses caused by the consumption of contaminated food annually. This situation leads to approximately 420,000 fatalities and a loss of 33 million healthy life years (WHO, 2024; Shahnia and Khaksar, 2013). Health researchers and food manufacturers are increasingly concerned about the proliferation of foodborne diseases attributed to bacterial contamination. The absence of preservatives can facilitate microbial growth in food products, thereby heightening the risk of food poisoning. In recent years, the adverse effects associated with synthetic preservatives such as allergic reactions, headaches, cancer, heart palpitations, and damage to the kidneys and liver have led to a growing awareness among consumers regarding their potential dangers. Consequently, many individuals now favor natural preservatives derived from plant sources, as well as those obtained from microbial and animal origins (Khan *et al.*, 2024; Matos *et al.*, 2013; RasGele and KaymaK, 2013; Rathee *et al.*, 2023; Shariat *et al.*, 2017). Algae represent a significant natural resource whose derivatives can serve as effective food preservatives. These photosynthetic organisms exist in both unicellular and multicellular forms within aquatic environments (Arica *et al.*, 2017). Annually, approximately 12 million tons of algae are cultivated, with around 85% of this quantity being utilized for human consumption and various food products (Food and Nations, 2018). In comparison to terrestrial animal products and plants, seaweeds are rich in minerals, essential fatty acids, and vitamins, including A, B, C, and Omega 3. For example, Dixit and Reddy (2017) reported that the total macro and micro minerals in the dry weight of Jania rubens amounted to 18,948.89 ± 1.06 mg per 100 grams. Furthermore, they exhibit therapeutic benefits such as promoting weight loss, reducing blood pressure and lipid levels, and acting as laxatives. Notably, algae have been associated with the prevention of atherosclerosis and the healing of gastrointestinal ulcers (Mendes *et al.*, 2013; Wijesekara *et al.*, 2011). The antimicrobial effect of different green, brown and red algae has been investigated and proven in various studies (Karthick *et al.*, 2019; Khezri Ahmad Abad *et al.*, 2016; Maharini *et al.*, 2022; Moubayed *et al.*, 2017; Ravi *et al.*, 2019; Zobeidy Nezhad *et al.*, 2018). Algae extract is widely used in tablets, ready -to-eat snacks (De Marco *et al.*, 2014), food formulations such as jelly, capsules, gums, pasta and beverages such as milk (Gouveia *et al.*, 2007). Algal biomass production can be used alongside technology for applications in superfoods (Ranga Rao and Ravishankar, 2018). In almost 400 genera of red algae, 3,900 species were identified, most of which are marine. Red algae can be used in the production of pigments, arachidonic acid, carrageenan and agar (Sharifian *et al.*, 2019). *Jania rubens*, a red macroalga from the *Corallinaceae* family, exhibits a distinctive structural composition. This species is found in various marine environments, including the Black Sea, Mediterranean Sea, Northeast Atlantic, China Sea, and Indian Ocean. Research indicates that the aqueous extract derived from *Jania rubens* possesses notable antimicrobial and antifungal properties (Karabay-Yavasoglu *et al.*, 2007). The algae *Jania rubens* serves as a valuable source for the extraction of xylogalactan, a compound that finds industrial application as a thickening agent and gel-forming substance. Additionally, extracts from this alga are utilized in cosmetic formulations, where they contribute to collagen synthesis aimed at reducing the appearance of cellulite and enhancing fat elimination (Dixit and Reddy, 2017). Ismail-Ben Ali *et al.* (2018,2012) conducted a study on the antimicrobial properties of *Jania rubens* extract, concluding that summer is the optimal season for harvesting this alga to maximize the production of secondary metabolites, particularly effective against *Streptococcus* and *Staphylococcus aureus*. The researchers isolated 19 epiphytic bacterial strains from the surface of the algae, with 7 demonstrating notable antimicrobial activity. Given Iran's diverse marine ecosystems and various algal species, there exists significant potential for the extraction of bioactive compounds from algae for application in multiple industries. Consequently, this study aims to identify the ideal extraction parameters for *Jania rubens* and to evaluate the antimicrobial efficacy of the resulting extract against foodborne pathogenic bacteria.

2. Materials and Methods

2.1 Sample preparation

The algae species *Jania rubens* was acquired from Fars Algae Biological Resources Development Company. Subsequently, it was subjected to drying in an oven (Metrohm model, Switzerland) at a controlled temperature of 50 degrees Celsius for 24 hours and then finely ground using an electric mill (Zarin Taj model, Iran). Microbial strains including *Escherichia coli, Staphylococcus aureus, Salmonella enteritidis,* and *Bacillus cereus* were acquired from the Iran Scientific and Industrial Research Organization.

2.2 Extraction procedure

The extraction process was conducted using the immersion method at a 10% mass-volume ratio, employing solvents i.e. 60% methanol, acetone, and a mixture of n-hexane, diethyl ether, and chloroform (Merk Co.). Initially, 20 grams of dried algae powder were measured and placed into a 50 ml cartridge. Subsequently, 200 ml of the solvent was added to a round-bottom flask, and Soxhlet extraction was carried out for durations specific to each solvent. Following this, the extracts of methanol, acetone, and

the hexane-diethyl ether-chloroform mixture were concentrated using a rotary evaporator (Laborota, Switzerland) and subsequently dried under a laminar flow hood. The resulting extracts were then reconstituted in dimethyl sulfoxide (Merk Co.) to achieve a concentration of 0.2 mass-volume, after which each extract was filtered through 0.45-micron diameter filters (Hajimehdipoor *et al.*, 2009).

2.3 Investigation of antimicrobial activity of *Jania rubens* extract

The evaluation of the antimicrobial properties of algae extract was conducted by assessing the diameter of the zone of inhibition (ZOI) against bacterial strains, utilizing the agar well diffusion technique. Initially, a microbial suspension was prepared to achieve a half McFarland turbidity standard (1.5 × 10^8 colony-forming units/ml), followed by the application of a sterile swab to uniformly inoculate the surface of the culture medium. Subsequently, wells measuring 6 mm in diameter were formed on the culture medium's surface using the end of a sterile Pasteur pipette. Each well was then inoculated with 20 microliters of thawed culture medium, and the plates were refrigerated for 15 minutes. Following this, 50 microliters of each algae extract were introduced into the designated wells, and the plates were incubated in a Jal Tehiz model incubator (Iran) at 37 degrees Celsius for a duration of 24 hours. After incubation, the sensitivity or resistance of the bacterial strains to the extract was determined by measuring the ZOI (Jahangirian *et al.*, 2013; Mashhadinejad *et al.*, 2016). The assessment of viable microorganisms within a specified volume of microbial culture medium exhibiting particular turbidity was conducted following the methodology established by Hedges (2002). Initially, a 24-hour culture was cultivated for each bacterial strain. Subsequently, a 1% superculture medium was prepared, and a 16-hour culture—tailored to the specific bacterial strain—was utilized to ascertain the colony-forming units. Following the measurement of turbidity in the 16-hour culture, the serial dilution technique was employed. After preparing the serial dilutions, a volume of 100 microliters was transferred to an agar culture medium plate (Merk Co.) and incubated for a duration of 24 hours. Upon completion of the incubation period, the colonies were enumerated, and the count was adjusted according to the dilution factor to determine the bacterial concentration in the original volume.

2.4 Assessment of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The susceptibility of bacteria to the extracts was evaluated through the serial dilution method in wells. Initially, 100 microliters of Mueller Hinton Broth (Merk Co.) were dispensed into seven wells. Subsequently, 100 microliters of the diluted extract solution were introduced into the first well. After thorough mixing, 100 microliters were transferred from the first well to the second well, and this process continued sequentially until the final well. At this point, 100 microliters of the culture medium were extracted from the last well, and 5 microliters of a microbial suspension with a concentration of 1.5 x 10^6 units/ml was added. The plates were then incubated at 37°C for 24 hours. The first well exhibiting inhibition of bacterial growth post-incubation was identified as the least inhibited. To ascertain clarity in the wells, 10 microliters were sampled and inoculated onto Mueller Hinton Agar (Merk Co.), and after 24 hours, the first dilution that achieved a 99.99% reduction in bacterial viability was designated as the minimum lethal concentration (Sabbagh *et al.*, 2015).

2.5 The effect of algae extracts on the growth of target bacteria

A nutrient agar medium was employed to assess the influence of the extract on the growth of targeted bacteria. To ensure temperature uniformity and adequate oxygen mixing, the flasks containing the culture medium were placed in a shaker set at 37°C and 200 RCF one day prior to the experiment. A loop of pure bacterial culture was inoculated into the nutrient liquid culture medium a day in advance and maintained under identical conditions as the other Erlen flasks. The following day, 0.3 ml of the bacterial culture medium was evenly distributed into the remaining Erlen flasks. Subsequently, extracts for the designated concentrations—minimum inhibitory concentration, half of the minimum inhibitory concentration, a quarter of the minimum inhibitory concentration, and a control—were prepared for each Erlen flask. The concentration range was determined based on the results of the minimum inhibitory concentration test. Additionally, control Erlen flasks containing bacteria without extract and those containing extract without bacteria were included as positive and negative controls, respectively. Every two hours, the turbidity of the culture medium in each Erlen flask was measured using a spectrophotometer (Thermo model, Germany), and the corresponding growth curve was plotted (Buch and Rollová, 2019).

2.6 Experimental Design

The experimental design was developed utilizing Design Expert software version 7.0.0, employing the Box-Behnken response surface methodology alongside a quadratic model. This design took into account three factors: the type of solvent, the duration of extraction, and the ratio of solvent to algae sample, as detailed in Table 1. A total of 13 experimental runs were established for the extraction process, as presented in Table 2. In this design, solvents were ranked based on their polarity and dielectric constant, utilizing the Dummy coding technique.

Table 1: Variables of interest in the extraction of compounds from *Jania rubens* algae using the Soxhlet method.

Factor	Lower limit	Upper Limit	code
Solvent	*	*	A
Time (Hour)	6	2	B
Solvent/Sample Ratio	10:1	5:1	D

* Solvent 1: Acetone (dielectric constant 0.32), Solvent 2: n-hexane + diethyl ether + chloroform (dielectric constant < 15), Solvent 3: methanol (dielectric constant 32.7).

Table 2: Experiment design using Design Expert software and Box-Behnken design method based on three factors: type of solvent, time, and ratio of solvent to sample for *Jania rubens* algae.

Run	(%w/v) Solvent/Sample	Time (Hour)	Solvent
1	5	2	3
2	2.5	2	2
3	5	6	3
4	2.5	4	2
5	5	2	2
6	10	4	2
7	5	6	3
8	10	4	1
9	5	6	2
10	10	4	2
11	5	2	2
12	10	2	1
13	10	6	2

Solvent 1: Acetone, Solvent 2: n-hexane + diethyl ether + chloroform, Solvent 3: methanol

3. Results and Discussion

3.1 Zone of Inhibition

To assess the antimicrobial properties of methanolic, acetone, and mixed extracts (n-hexane + diethyl ether + chloroform) derived from the examined algae, the diameter of the zone of inhibition (ZOI) was measured for the extracts. The findings are presented in Table 3. A larger zone of inhibition (ZOI) signifies enhanced antibacterial effectiveness, whereas a smaller ZOI indicates restricted activity. *Staphylococcus aureus* exhibits the highest maximum ZOI at 20.97 mm, reflecting a strong antibacterial effect against this particular pathogen. However, its minimum ZOI of 8.30 mm points to variability in its effectiveness, which may be influenced by the concentration of the agent tested. *Escherichia coli* closely follows, with a maximum ZOI of 16.47 mm, demonstrating considerable antibacterial potential. Its minimum ZOI of 10.11 mm suggests a more consistent effectiveness, albeit less pronounced than that of Staphylococcus aureus. *Bacillus cereus* presents moderate results, with a maximum ZOI of 14.80 mm and a minimum of 8.95 mm, indicating effective but somewhat inconsistent antibacterial action, potentially due to variability in strain resistance or response to the tested agent. *Salmonella enteritidis* shows the smallest range, with a maximum ZOI of 12.08 mm and a minimum of 6.50 mm, suggesting that while there is some antibacterial activity, it is significantly lower compared to the other pathogens, indicating a higher level of resistance or the necessity for more potent agents to achieve substantial inhibition.

3.2 Determining the optimal extraction conditions based on antimicrobial activity

The findings derived from the data analysis concerning the two measured factors, MBC and MIC, indicate that the suggested models for examining the influence of three independent variables—namely, solvent type, extraction time, and solvent ratio employed in the extraction process—are as follows.

Bacillus cereus

MIC

The findings derived from the data analysis concerning the measurement of the MIC factor for *Bacillus cereus* indicate that the model proposed is linear in nature. The output of the one-way ANOVA for the linear model of the response level are presented in Table 4.

MBC

The results of the analysis of data obtained from measuring the MBC indicated that the proposed model is a linear model. Table 5 presents the results of the one-way ANOVA for the response surface linear model.

Table 3: comparative analysis of the maximum and minimum diameters of the zone of inhibition (ZOI) observed in the target bacterial strains.

ZOI	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella enteritidis*	*Bacillus cereus*
min	10.11	8.3	6.5	8.95
max	16.47	20.97	12.08	14.8

Table 4: The results of the ANOVA conducted on the linear model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.45	3	0.4839	53.42	**< 0.0001**	significant
A-Solvent	1.42	1	1.42	157.08	**< 0.0001**	
B-Time	0.0630	1	0.0630	6.96	0.0270	
C-Percent	0.0004	1	0.0004	0.0389	0.8480	
Residual	0.0815	9	0.0091			
Lack of Fit	0.0815	8	0.0102			
Pure Error	0.0000	1	0.0000			
Cor Total	1.53	12				

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

In Formula 1, the relationship between MIC and the investigated factors is presented.

MIC= 0.821465 + 0.402121 * A + -0.0702525 * B + -0.00262626 * C ----- ***Formula 1***

Figures 1 and 2 illustrate the correlation among factor B, factor A, and the MIC value as represented by a linear equation.

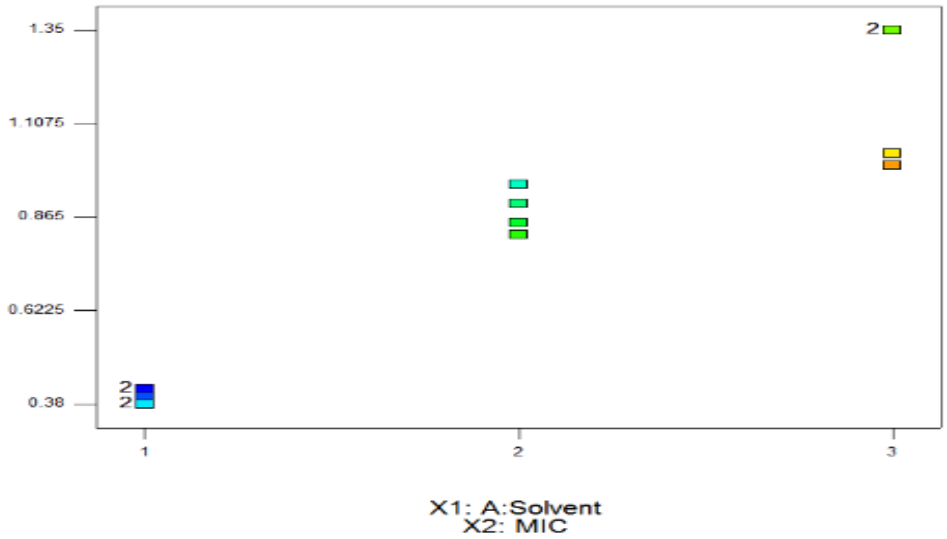

Fig. 1: The relationship between the type of solvent used and the MIC value.

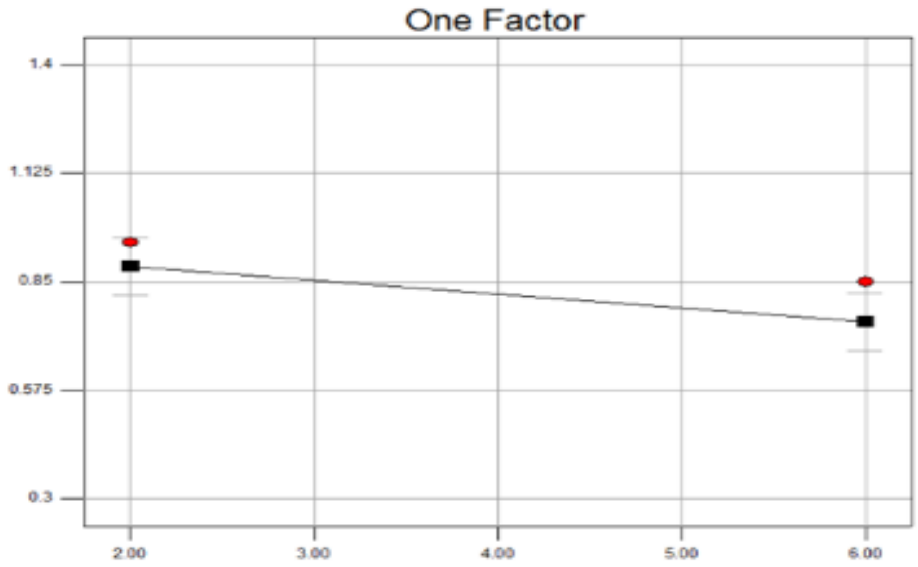

Fig. 2: The relationship between the time of extraction and the MIC value.

Table 5: The results of the ANOVA conducted on the linear model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.53	3	0.5087	24.50	**0.0001**	significant
A-Solvent	1.42	1	1.42	68.33	**< 0.0001**	
B-Time	0.1709	1	0.1709	8.23	**0.0185**	
C-Percent	0.0000	1	0.0000	0.0006	0.9806	
Residual	0.1869	9	0.0208			
Lack of Fit	0.1869	8	0.0234			
Pure Error	0.0000	1	0.0000			
Cor Total	1.71	12				

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Formula 2 illustrates the correlation between the factors under investigation and the MBC factor.

MBC=1.19747 + 0.401515 * A + -0.115657 * B + 0.000505051 * C

--- ***Formula 2***

In Figures 3 and 4, the relationship between the type of solvent used and the amount of MBC is shown in the linear equation.

Escherichia coli

MIC

The findings derived from the data analysis concerning the measurement of the MIC for *Escherichia coli* indicate that the proposed model adheres to a linear framework. The outcomes of the ANOVA for the linear model of the response level are detailed in Table 6. Formula 3 illustrates the correlation between the factors under investigation and the MIC factor.

MIC=1.15284 + 0.275227 * A + -0.0773485 * B + -0.00450758 * C

----Formula 3

Additionally, Figures 5 and 6 depict the association between the two factors, A and B, and the MIC value as represented in the linear equation.

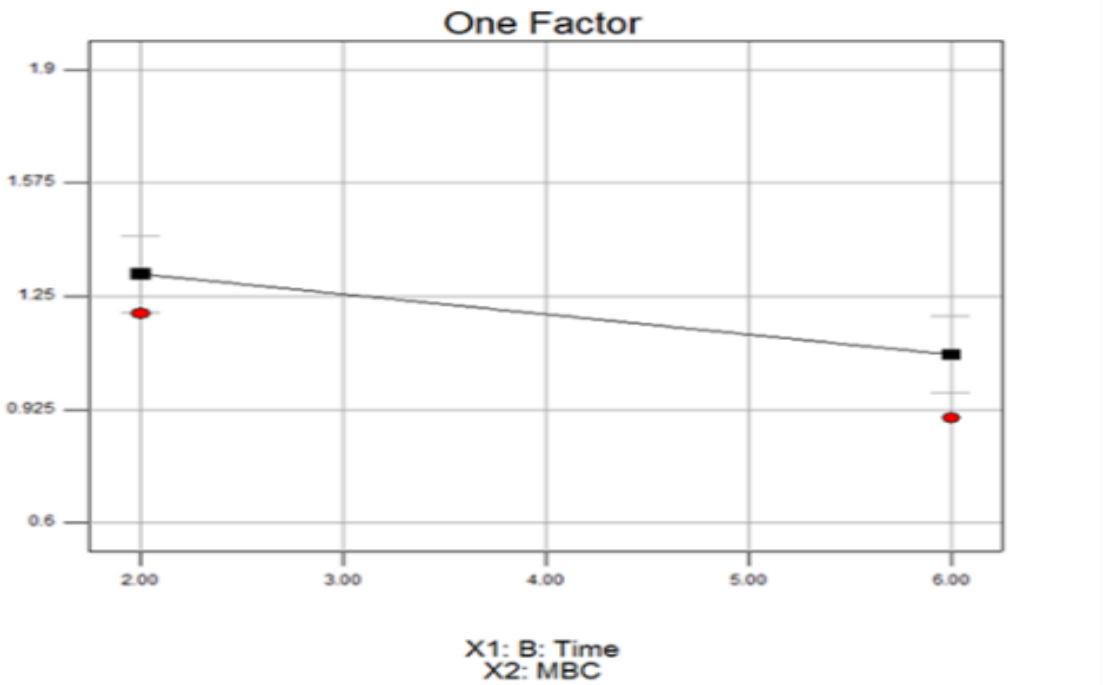

Fig. 3: The relationship between the time of extraction and the MBC value.

Table 6: The outcomes of the one-way analysis of variance pertaining to the linear model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.7117	3	0.2372	86.75	**< 0.0001**	significant
A-Solvent	0.6666	1	0.6666	243.75	**< 0.0001**	
B-Time	0.0764	1	0.0764	27.95	**0.0005**	
C-Percent	0.0010	1	0.0010	0.3796	0.5531	
Residual	0.0246	9	0.0027			
Lack of Fit	0.0242	8	0.0030	6.71	0.2904	not significant
Pure Error	0.0005	1	0.0005			
Cor Total	0.7363	12				

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

MBC

The findings derived from the data analysis concerning the measurement of the (MBC) factor for *Escherichia coli* indicate that the model proposed is linear in nature. The outputs of the ANOVA for the linear model of response levels are presented in Table 7.

In formula 4, the relationship between the examined factors and the MBC factor is presented.

MBC= 2.57071 + 0.337879 * A + -0.0914141 * B + -0.0040404 * C

---- Formula 4

Figures 7 and 8 show the relationship between the B, A factor and the MBC value in the linear equation.

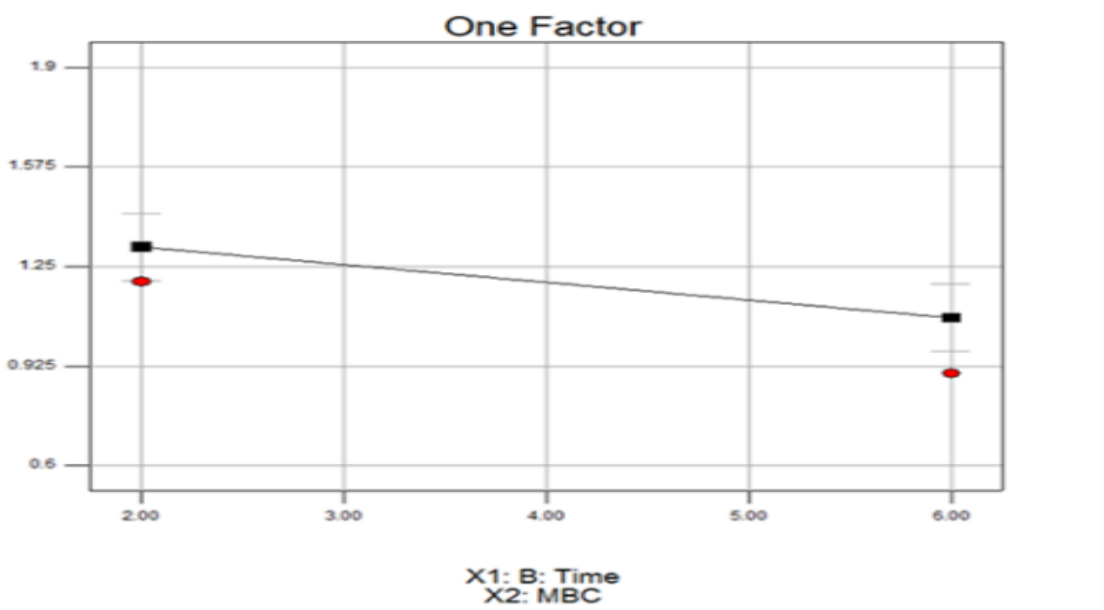

Fig. 4: The relationship between the type of solvent used and the MBC value.

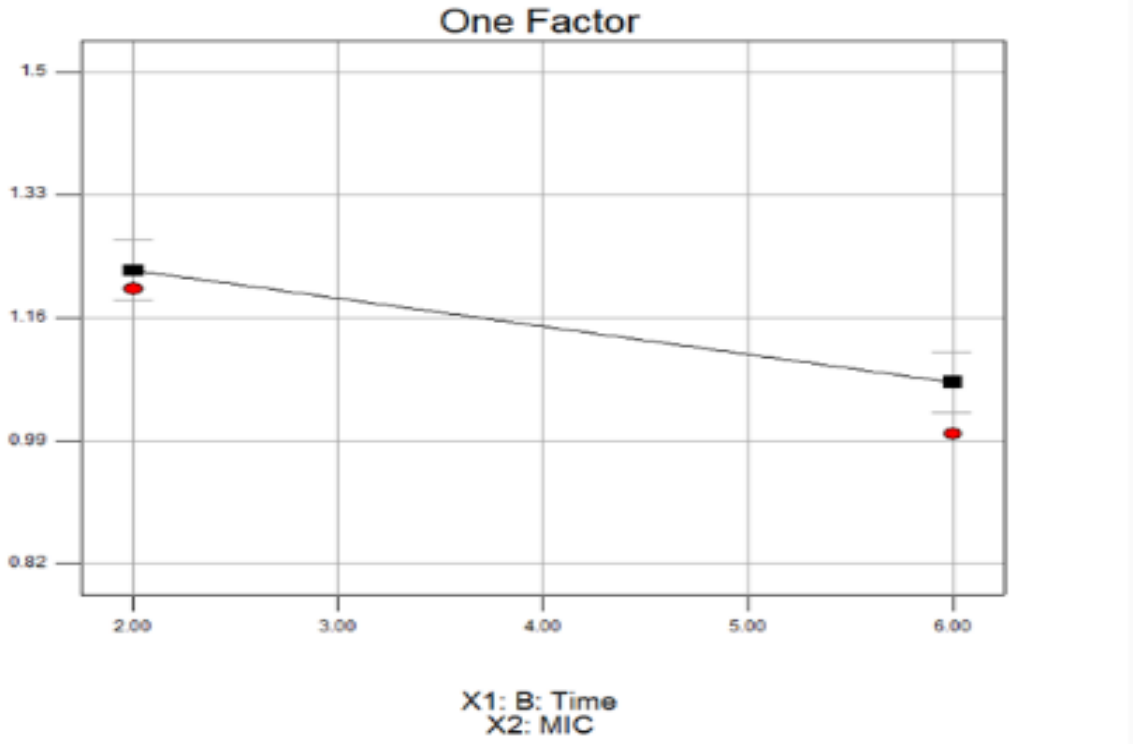

Fig 5. The relationship between the time of extraction and the MIC value.

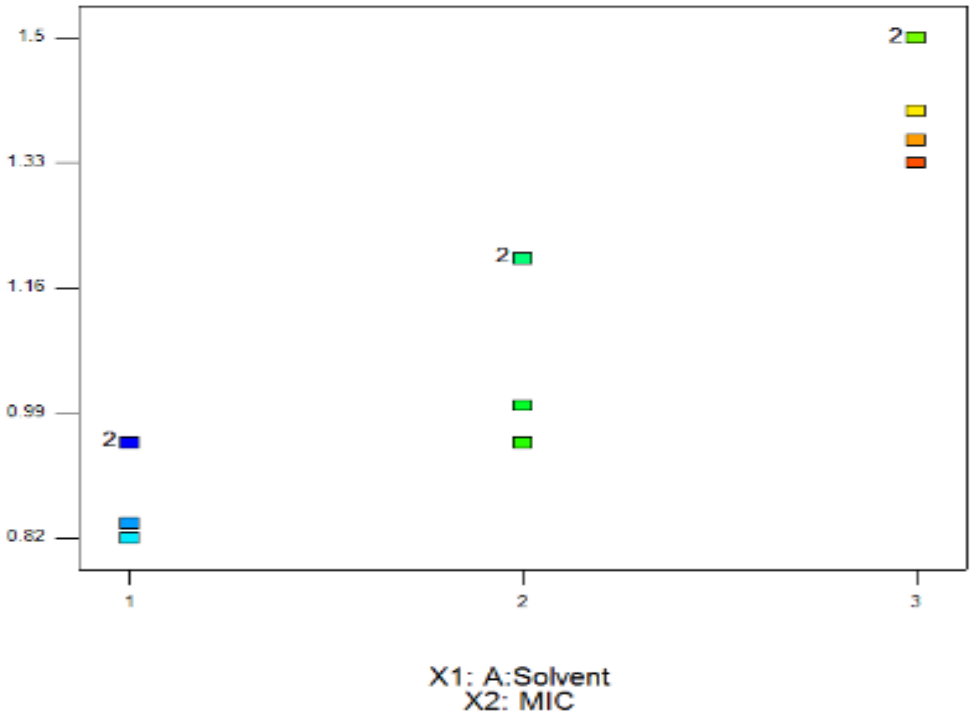

Fig. 6: The relationship between the type of solvent used and the MIC value.

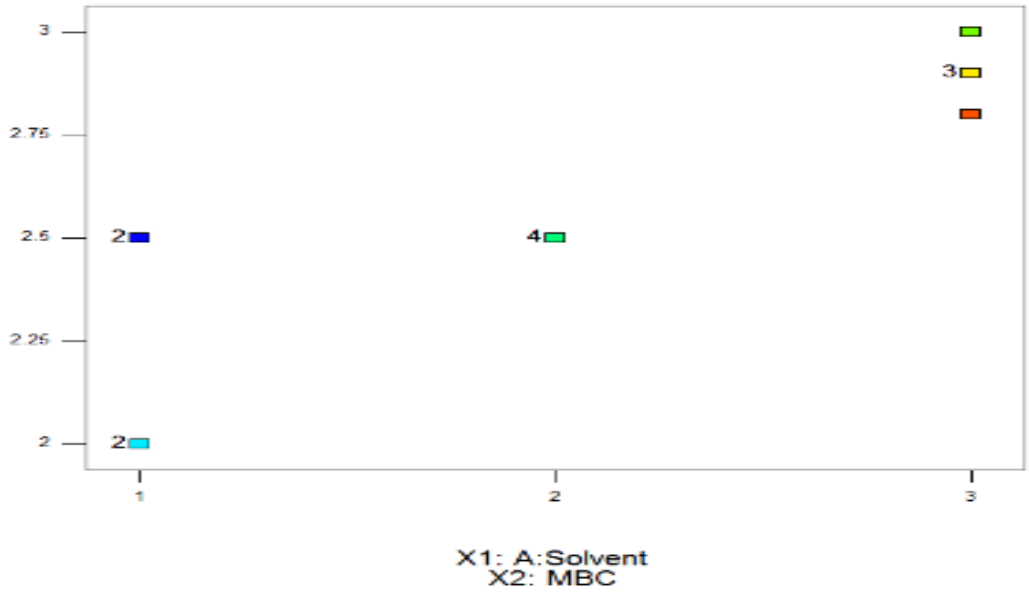

Fig. 7: The relationship between the type of solvent used and the MBC value.

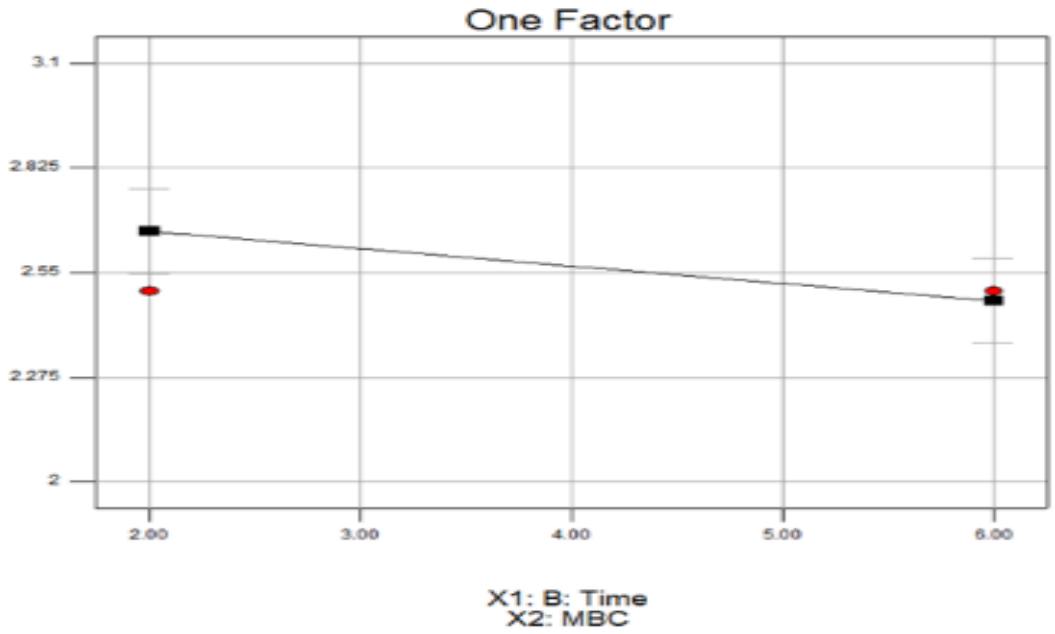

Fig. 8: The relationship between the time of extraction and the MBC value.

Staphylococcus aureus

MIC

The results of data analysis obtained from the measurement of MIC factor for Staphylococcus aureus factor showed that the proposed model is a linear model. Table 8 shows the results of ANOVA for the response level linear model.

In formula 5, the relationship between the examined factors and the MIC factor is presented.

MIC=0.853333 + 0.45125 * A + -0.042 * B + 0.000666667 * C

--- Formula 5

Figures 9 and 10 show the relationship between the B and A factor and the MIC value in the linear equation.

MBC

The results of data analysis obtained from the measurement of MBC factor for *Staphylococcus aureus* showed that the proposed model is a linear model. Table 9 shows the results of ANOVA for the response level linear model.

In formula 6, the relationship between the investigated factors and the MBC factor is presented.

MIC=1.40778 + 0.905 * A + 0.0455556 * B + 0.0244444 * C

--- Formula 6

Figure 11 shows the relationship between factor A and MBC value in the linear equation.

Salmonella enteritidis

MIC

The results of the analysis of the data obtained from the measurement of the MIC factor for *Salmonella enteritidis* showed that the proposed model is a linear model. Table 10 shows the results of ANOVA for the response level linear model.

In formula 7, the relationship between the investigated factors and the MIC factor is presented.

1/MIC=0.761086 + -0.206745 * A + 0.0287789 * B + -0.00096055 * C

---formula 7

In figures 12 and 13, the relationship between factor A and B of the MIC value is displayed in the linear equation.

MBC

The results of analyzing the data obtained from measuring the MBC factor for *Salmonella enteritidis* showed that the proposed model is a linear model. Table 9 shows the results of ANOVA for the linear model of the response level.

In Formula 8, the relationship between the investigated factors and the MBC factor is presented

MBC=3.10227 + 0.336364 * A + -0.0742424 * B + -0.00378788 * C

---Formula 8

Figure 14 shows the relationship between factor A and MBC value in the linear equation.

Table 7: Results of analysis of variance pertaining to the linear model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.07	3	0.3556	18.16	**0.0004**	significant
A-Solvent	1.00	1	1.00	51.30	**< 0.0001**	
B-Time	0.1067	1	0.1067	5.45	0.0444	
C-Percent	0.0008	1	0.0008	0.0426	0.8411	
Residual	0.1763	9	0.0196			
Lack of Fit	0.1713	8	0.0214	4.28	0.3582	not significant
Pure Error	0.0050	1	0.0050			
Cor Total	1.24	12				

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table 8. The results of analysis of variance of the linear model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.65	3	0.5506	29.66	**< 0.0001**	significant
A-Solvent	1.63	1	1.63	87.77	**< 0.0001**	
B-Time	0.0227	1	0.0227	1.22	0.2976	
C-Percent	0.0000	1	0.0000	0.0012	0.9728	
Residual	0.1670	9	0.0186			
Lack of Fit	0.1670	8	0.0209	5.48	0.0678	insignificant
Pure Error	0.0001	1	0.0001			
Cor Total	1.82	12				

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

Table 9: ANOVA analysis results of the linear model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6.61	3	2.20	5.47	**0.0204**	significant
A-Solvent	6.55	1	6.55	16.25	**0.0030**	
B-Time	0.0267	1	0.0267	0.0662	0.8027	
C-Percent	0.0307	1	0.0307	0.0762	0.7887	
Residual	3.63	9	0.4031			
Lack of Fit	3.63	8	0.4535			
Pure Error	0.0000	1	0.0000			
Cor Total	10.24	12				

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

Table 10: Results of analysis of variance of the linear model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.4023	3	0.1341	100.10	**< 0.0001**	significant
A-Solvent	0.3761	1	0.3761	280.76	**< 0.0001**	
B-Time	0.0106	1	0.0106	7.90	**0.0204**	
C-Percent	0.0000	1	0.0000	0.0352	0.8554	
Residual	0.0121	9	0.0013			
Lack of Fit	0.0116	8	0.0015	3.40	0.3976	not significant
Pure Error	0.0004	1	0.0004			
Cor Total	0.4144	12				

P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

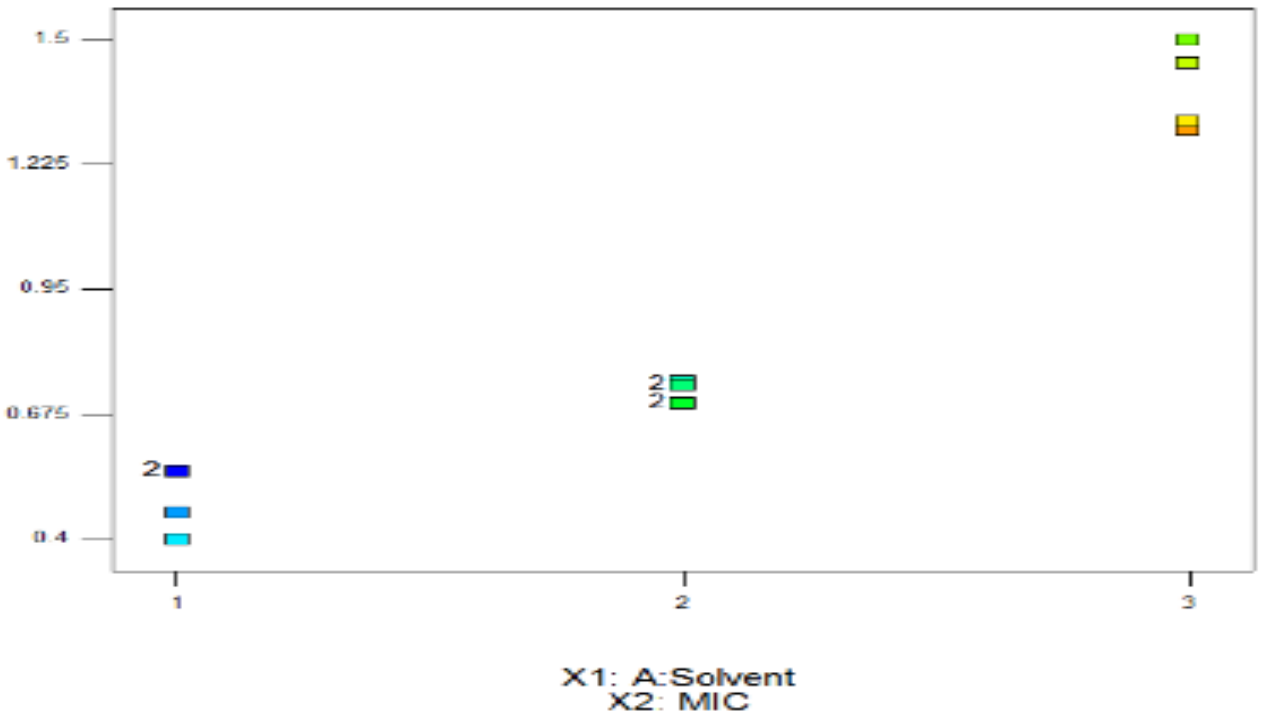

Fig. 9: The relationship between the type of solvent used and the MIC value.

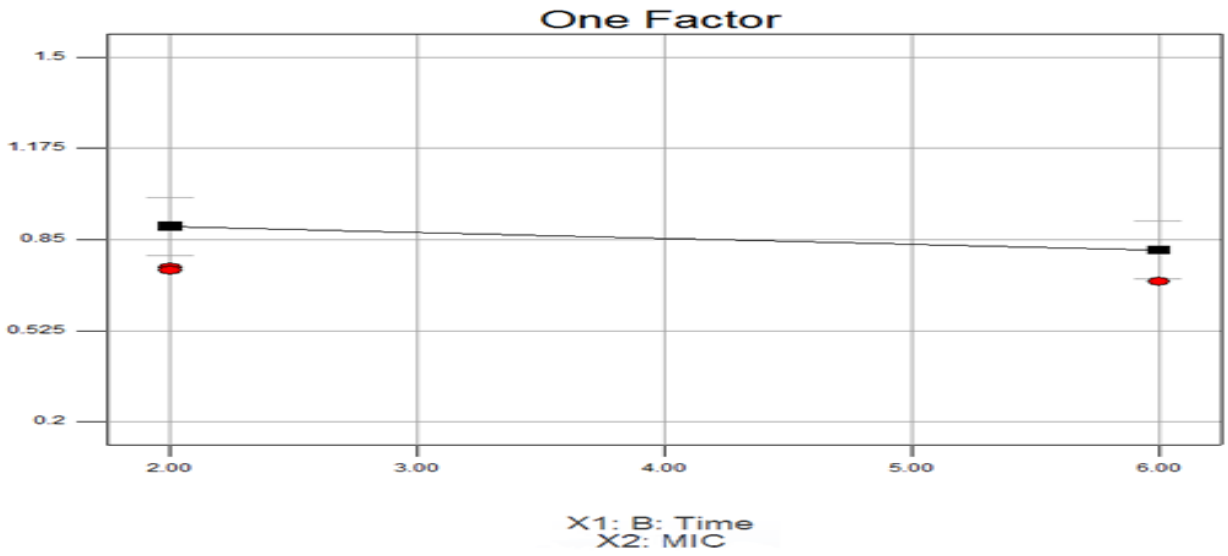

Fig. 10: The relationship between the time of extraction and the MIC value.

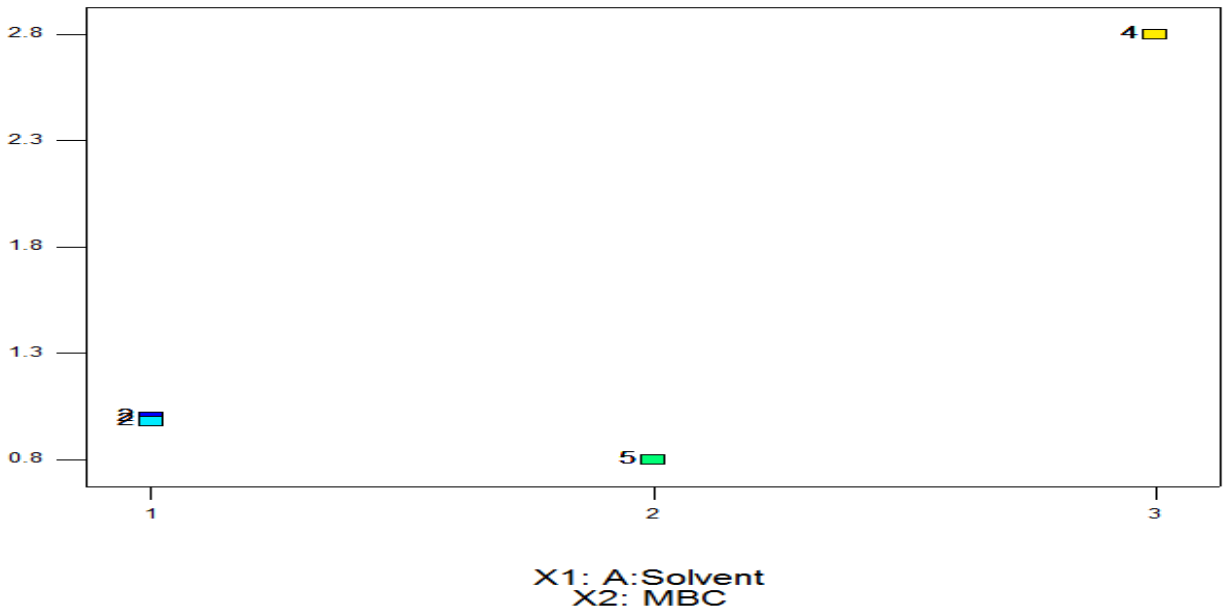

Fig. 11: The relationship between the type of solvent used and the MBC value.

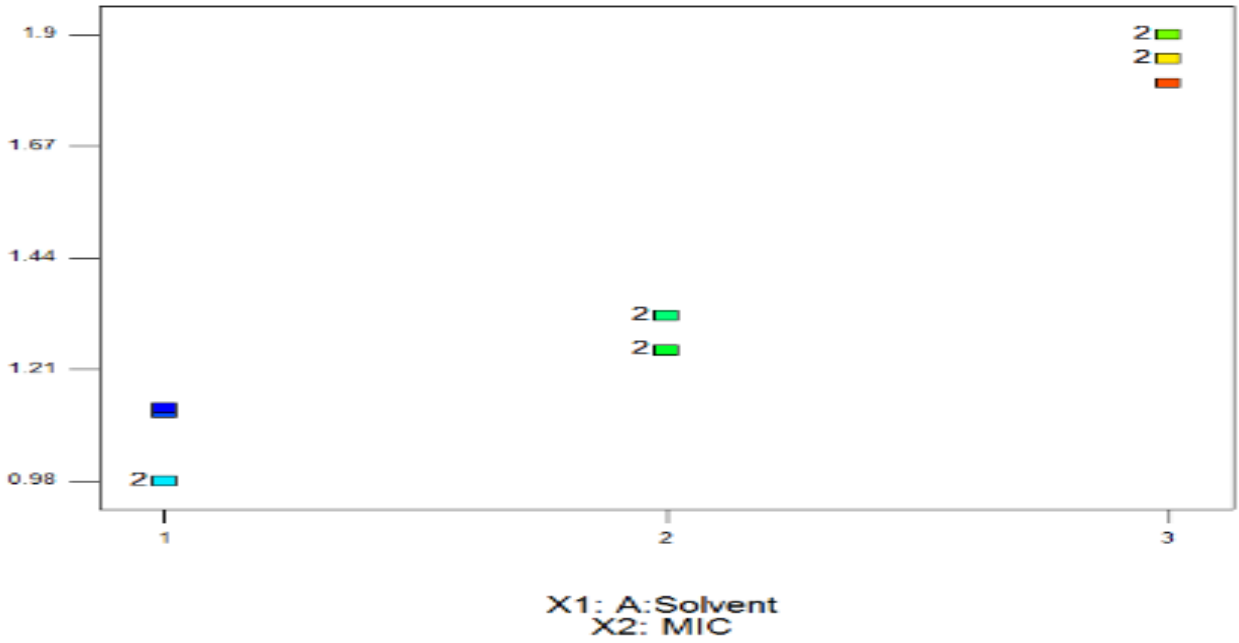

Fig. 12: The relationship between the type of solvent used and the MIC value.

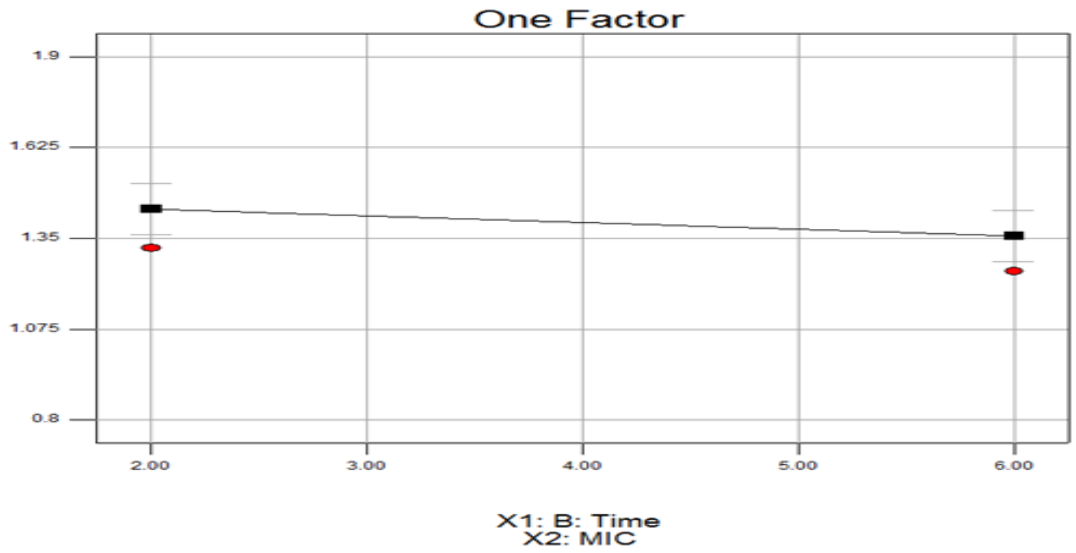

Fig. 13: The relationship between the time of extraction and the MIC value.

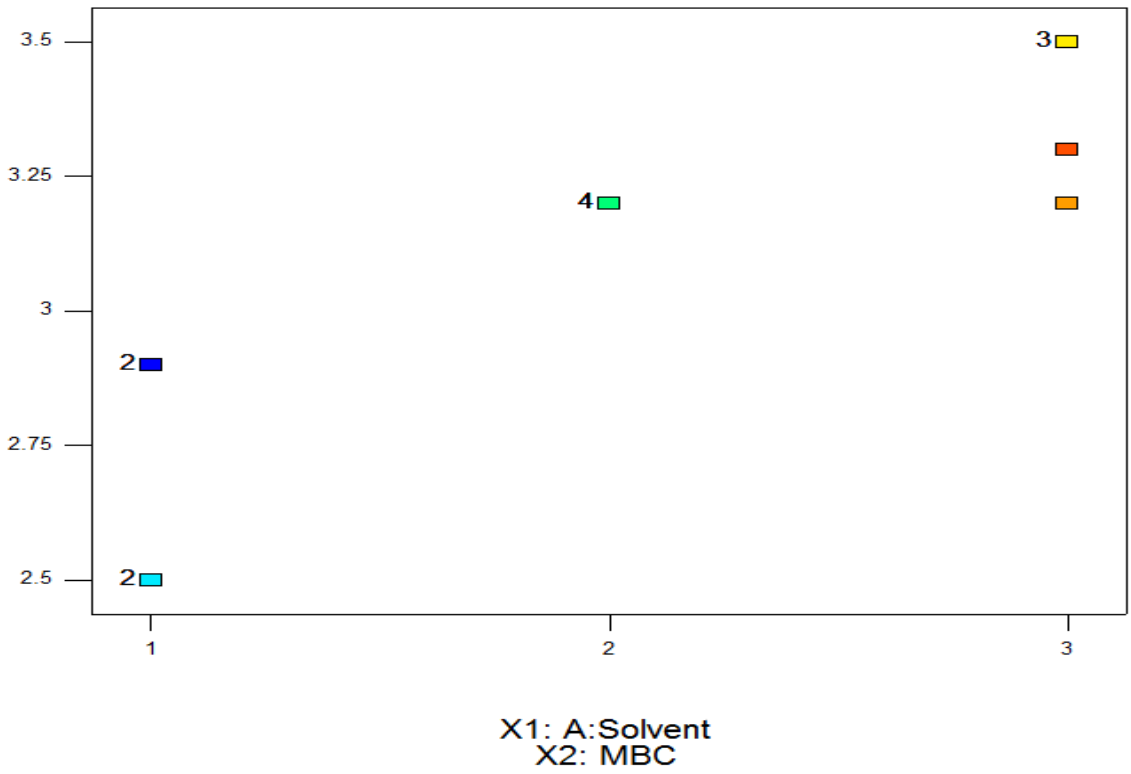

Fig. 14: The relationship between the type of solvent used and the MBC value.

Table 11: Results of analysis of variance of the linear model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.13	3	0.3778	14.80	**0.0008**	significant
A-Solvent	0.9956	1	0.9956	39.01	**0.0002**	
B-Time	0.0704	1	0.0704	2.76	0.1311	
C-Percent	0.0007	1	0.0007	0.0287	0.8692	
Residual	0.2297	9	0.0255			
Lack of Fit	0.2097	8	0.0262	1.31	0.5922	not significant
Pure Error	0.0200	1	0.0200			
Cor Total	1.36	12				

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

Table 12: Optimized condition for the extraction of compounds from algae utilizing the Soxhlet method, demonstrating efficacy against *Escherichia coli.*

Number	Solvent	Time	Percent	MIC	MBC	Desirability	
1	1	6.00	10	0.796	2.137	**0.929**	Selected

Table 13: Optimized condition for the extraction of compounds from algae utilizing the Soxhlet method, demonstrating efficacy against *Staphylococcus aureus.*

Number	Solvent	Time	Percent	MIC	MBC	Desirability	
1	1.000	6.000	10.000	0.361	0.573	**1.000**	Selected

Table 14: comparative analysis of the minimum and maximum values for MIC, MBC, and ZOI for four bacterial strains exposed to *Jania rubens extract*

Bacterial Strain	MBC	MIC
	min	min
	max	max
Escherichia coli	2	0.82
	3	1.5
Staphylococcus aureus	0.8	0.4
	2.8	1.5
Salmonella enteritidis	2.5	0.98
	3.5	1.9
Bacillus cereus	0.85	0.38
	1.85	1.35

The statistical analysis conducted indicates that two bacterial strains, ***Escherichia coli and Staphylococcus aureus***, were chosen to examine the influence of *Jania rubens* algae extract, which was prepared under optimal conditions as detailed in the subsequent tables, on their growth curves.

The optimal extraction conditions, as determined by the MIC and MBC values, involve the use of solvent number (1), which is acetone with medium polarity. The extraction was conducted for a duration of 6 hours with a solvent-to-sample ratio of 10. Additionally, another set of conditions with the same solvent number (1) and medium polarity (acetone) was applied, but with an extraction time of 2 hours and a solvent-to-sample ratio of 10. Table 12 presents a comparative analysis of the minimum and maximum values for the three factors: MIC, MBC, and the diameter of the zone of inhibition (ZOI) for four bacterial strains in relation to the extracts derived from the red algae *Jania rubens.*

Growth curve of *Escherichia coli* and *Staphylococcus aureus* in the presence of *Jania rubens* extract

To ascertain the impact of *Jania rubens* algae extract on the growth profiles of pathogenic bacteria, *Escherichia coli and Staphylococcus aureus*, the absorption intensity of these bacteria was monitored over a 24-hour period. The growth curve of *Escherichia coli and Staphylococcus aureus* in the presence of *Jania rubens* algae extract are illustrated in Figures 15 and 16.

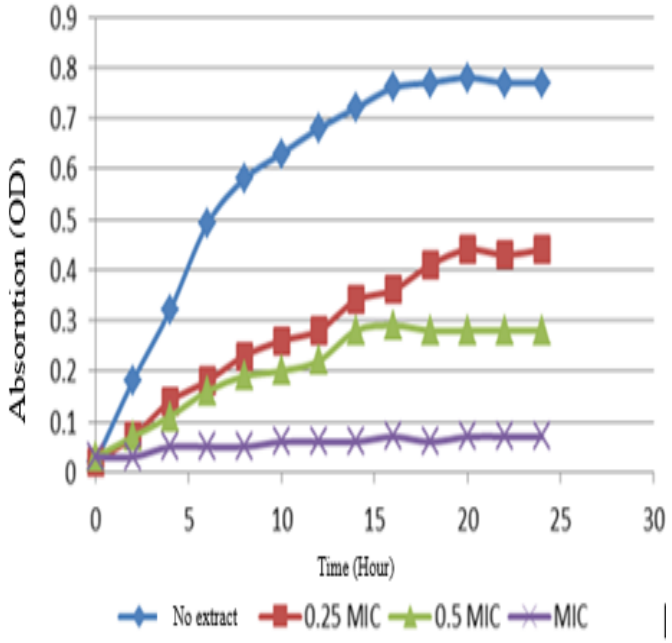

Fig. 15: The growth curve of *Escherichia coli during 24 hours*

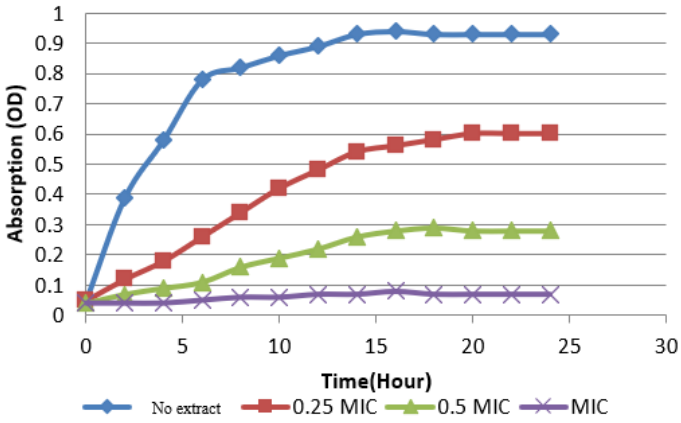

Fig. 16: The growth curve of *Staphylococcus aureus during 24 hours*

The data presented in the graphs indicate that the control samples of pathogenic bacteria exhibit a lag phase of approximately 2 hours. However, this lag phase is significantly prolonged when exposed to the extract of *Jania rubens* algae. Specifically, in samples treated with a concentration corresponding to one-quarter of the minimum inhibitory concentration (MIC 0.25) of *Jania rubens* extract, the lag phase for pathogenic bacteria extended to roughly 4 hours. Furthermore, in samples subjected to a concentration equivalent to half of the minimum inhibitory concentration (MIC 0.5), a delay phase of 6 hours was recorded for the pathogenic bacteria. The degree of this increase was contingent upon the concentration of *the* extract; as the concentration of the extract rose, the length of the incubation phase also extended. In samples exposed to a concentration that matched the minimum inhibitory concentration of *Jania rubens* algae extract, the proliferation of the target bacteria was entirely halted, resulting in the absence of a lag phase for any of the target bacterial strains.

Following a duration of two hours in the control sample of pathogenic bacteria, there was a marked increase in absorption intensity coinciding with the onset of the growth phase, which subsequently stabilized upon reaching the stationary phase. In contrast, samples treated with *Jania rubens* algae extract exhibited a rise in absorption intensity, albeit at a reduced level compared to the control. Furthermore, the peak growth observed in these treated samples was inferior to that of the control. As illustrated in Figures 15 and 16, during the growth phase, the control samples of *Escherichia coli* and *Staphylococcus aureus* attained maximum absorption intensities of 0.77 and 0.94, respectively. Conversely, in samples subjected to a concentration equivalent to one-quarter of the minimum inhibitory concentration (MIC 0.25) of *Jania rubens* algae extract, the maximum absorption intensities for the tested bacteria diminished to 0.46 and 0.56. The observed reduction in concentration, corresponding to half of the minimum inhibitory

concentration (MIC 0.5) of algae extract, was measured at 0.29 and 0.32, respectively. These findings indicate that the growth curve of the bacteria exposed to extract exhibits a prolonged incubation period when compared to the control samples. Notably, the most significant difference in absorption intensity between the bacterial growth curves was recorded during both the growth and stationary phases for the control and treated samples. The data suggest that the bacteria demonstrate peak sensitivity to the *Jania rubens* algae extract during the growth phase, which subsequently diminishes in the resting phase.

The examination of the diameter of the zone of inhibition (ZOI) for various extracts of *Jania rubens* algae demonstrated that these extracts exhibited antimicrobial properties against the selected bacterial strains; however, they did not display uniform antimicrobial efficacy. Furthermore, the analysis of the minimum inhibitory concentration and the minimum lethal concentration of the different algae extracts indicated that the antimicrobial peptides varied in their effectiveness against the target bacteria. The study examining the antimicrobial properties of the aforementioned algae extracts against various bacterial strains revealed, based on the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assessments, that these extracts exhibited the highest antimicrobial efficacy against Gram-positive bacteria, specifically *Staphylococcus aureus* and *Bacillus cereus*. Conversely, the extracts demonstrated the least antimicrobial activity against Gram-negative bacteria, including *Escherichia coli* and *Salmonella enteritidis*. The increased resistance observed in Gram-negative bacteria can be attributed to the intricate structure of their cell wall. This structure not only comprises a cytoplasmic membrane but also features an outer membrane made up of phospholipids, lipopolysaccharides, lipoproteins, and proteins. The presence of these components in the outer membrane likely hinders the penetration of antibacterial extracts into Gram-negative bacteria (Bag *et al.*, 2012). These findings align with those of Kavita *et al.* (2014), who noted that Gram-negative bacteria exhibit greater resistance than Gram-positive bacteria when exposed to the methanolic extract of *Laurencia papillosa* algae. Furthermore, a separate investigation revealed that the ethanolic extract of *Jania rubens* algae, sourced from the southern coast of India, demonstrated significant antibacterial activity against both Gram-positive and Gram-negative bacteria; however, its efficacy was notably reduced against Gram-negative strains (Chenniappan *et al.*, 2021). Conversely, Mohammadi *et al.* (2016) reported contrasting results, indicating that while the brown algae *Iyengaria stellata* possesses antioxidant properties, its aqueous extract did not exhibit any antibacterial activity. (Lazzam and Ayal (2024) demonstrated that the methanolic extract of sargassum exhibits greater antibacterial efficacy compared to its aqueous counterpart, which failed to display any antibacterial activity against *Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Streptococcus mutans, Salmonella* species, and *Staphylococcus aureus*. The assessment of the MBC of various extracts from *Jania rubens* algae indicates that these values surpass the MIC. This suggests that the inhibitory effects of these algal extracts on bacterial growth are primarily due to the suppression of DNA replication, modulation of enzyme activity, and the damaging impact on the cytoplasmic membrane of the bacterial cells. However, to effectively halt bacterial proliferation, it is essential to utilize concentrations that exceed the lethal threshold of the extract (Kavita *et al.*, 2014). In initial experiments, the different extracts of *Jania rubens* demonstrated antimicrobial activity against all tested bacterial strains, as evidenced by the zone of inhibition (ZOI). Further analysis revealed that the minimum inhibitory concentrations and minimum lethal concentrations *for Escherichia coli* ranged from 0.32 to 1.5 and 3 to 2, respectively; for *Staphylococcus aureus*, the ranges were 0.4 to 1.5 and 0.8 to 2.8; for *Salmonella enteritidis*, they were 0.98 to 1.9 and 2.5 to 3.5; and for *Bacillus cereus*, the values were 0.36 to 1.35 and 0.85 to 1.85. The optimal extraction conditions identified include the use of a medium polarity solvent (acetone), an extraction duration of 6 hours, and a solvent-to-sample ratio of 5, as well as an alternative condition with the same solvent, a 2-hour extraction time, and the same solvent-to-sample ratio. In a report by Safari *et al.* in 2011, it was found that the acetone extract of the unicellular algae *Chlorella vulgaris* exhibited notable antimicrobial and growth-inhibitory effects against the gram-positive bacterium *Bacillus subtilis*, particularly when compared to its alcoholic extract. Conversely, research conducted by Mashhadinejad *et al.* in 2016 in 2016 indicated that the acetone extracts of green, red, and brown algae did not demonstrate inhibitory effects against the fungi *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Candida albicans*, and *Candida tropicalis*. Furthermore, in 2018, Sheikh *et al.* examined the antimicrobial properties of algae extracts sourced from the Red Sea shores in Jeddah, Saudi Arabia, revealing that green algae exhibited the most significant antifungal activity, followed by red and brown algae. The lowest inhibitory concentration of these algae extracts was reported to range from 0.5 to 4 mg/ml. In their 2022 study, Drishya and Medo MerinaMarina found that Gram-negative bacteria exhibited greater activity compared to Gram-positive bacteria when exposed to various extracts of *Sargassum Wightii* seaweed sourced from Tamil Nadu, India. The extracts obtained using methanol and chloroform demonstrated superior efficacy relative to those derived from other solvents. Specifically, the methanol and chloroform extracts inhibited *Proteus vulgaris*, while *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were inhibited by the chloroform extract, and *Escherichia coli* was affected by the ethyl acetate extract of *Sargassum Wightii*.

A separate investigation revealed that extracts from *Cystoseria mirica, Sargassum polycystome*, and *Turbina trichoetra* exhibited antimicrobial properties against both Gram-negative and Gram-positive bacteria (Mohammed, 2023). Furthermore, research conducted by Zamani Kochesfehani *et al.* in 2021) in 2021 demonstrated that the red algae *Gracilaria gracilis* possesses antibacterial compounds capable of inhibiting a wide range of bacterial species. The chemical compounds derived

from the three algae species, *Tetradesmus nygaardi*, *Scenedesmus quadricauda*, and *Coelastrella sp*., demonstrate significant efficacy against both gram-negative and gram-positive bacteria, leading to their inhibition or destruction. Notably, the ethanolic extract of *Scenedesmus quadricauda* exhibited a 95% greater effectiveness against *Acinetobacter baumannii, Klebsiella pneumoniae, Enterococcus faecalis, and Staphylococcus aureus* compared to the hexane extract. In contrast, the ethanolic extract of *Coelastrella sp*. displayed a comparatively weaker effect than the other algae examined (Toma and Aziz, 2023).

The growth curve of the target bacteria when exposed to the antibacterial extract derived from *Jania rubens* algae exhibits an extended incubation period in comparison to the control samples. This observation suggests that the compounds present in the bacterial growth medium may prolong the adaptation phase required for bacterial proliferation. It is likely that these antibacterial agents inhibit the production of essential mediators and enzymes that facilitate the initiation of bacterial growth. The enhancement of *Jania rubens* algae extract concentration correlates with a heightened impact on the communal phase. This suggests that the inhibitory efficacy of these compounds on bacterial enzyme synthesis and cellular metabolites is concentration-dependent, with their inhibitory strength escalating alongside increasing concentrations. Ultimately, at the minimum inhibitory concentration, these compounds exhibit a complete cessation of bacterial growth, resulting in the total absence of bacteria throughout the incubation period.

During the growth phase, following the adaptation of bacteria to their new environment and the synthesis of essential enzymes, there is a marked exponential increase in cell numbers. The rate of growth and the peak bacterial proliferation at this stage vary depending on the specific microorganism and the conditions of growth, such as temperature and the composition of the culture medium. Research indicates that as the concentration of *Jania rubens* algae extract rises, both the slope of the growth curve during the logarithmic phase and the peak bacterial growth, indicated by maximum turbidity, decline. This suggests that the antibacterial compounds present exert their inhibitory effects by interfering with DNA replication, disrupting cell wall division, and impacting enzymes critical for cell division. The most significant difference in absorption intensity between the bacterial growth curves of the control and treated samples was noted during both the growth and stationary phases. It appears that bacterial cells exhibit heightened sensitivity to chemical and physical influences during these phases (Babii *et al.*, 2016). As the concentration of antibacterial compounds increases, there is a corresponding decrease in the slope of the growth curve and maximum bacterial absorption, alongside an extension of the coexistence phase; furthermore, higher concentrations of these compounds correlate with intensified destructive effects.

Conclusion

This research successfully determined the optimal conditions for extracting antibacterial compounds from the algae Jania rubens utilizing the Soxhlet extraction technique. The study concentrated on key parameters including the type of solvent, duration of extraction, and the ratio of solvent to sample. The findings indicated that acetone emerged as the most effective medium-polarity solvent, when used for a 6-hour extraction period at a solvent-to-sample ratio of 5. The antimicrobial assessment of the extracts demonstrated notable inhibitory effects against Escherichia coli and Staphylococcus aureus, especially at concentrations below the minimum inhibitory concentrations (MIC) of 0.25 and 0.5, respectively. These results highlight the potential of Jania rubens as a promising source of natural antimicrobial agents, suggesting avenues for further investigation into its applications in food safety and public health. The outcomes not only deepen our understanding of the antimicrobial properties inherent in algal extracts but also aid in the formulation of alternative approaches for addressing bacterial infections, emphasizing the significance of refining extraction methods to enhance bioactivity.

The results of this study emphasize the promise of *Jania rubens* as a natural source of antimicrobial agents and stress the necessity for ongoing research in this domain. Subsequent investigations could focus on the isolation and characterization of particular bioactive compounds, which would facilitate a deeper understanding of their mechanisms of action and effectiveness against a wider array of pathogens. Furthermore, examining the potential uses of these extracts in food safety, pharmaceuticals, and other sectors may lead to novel approaches in microbial control and improvements in public health. In summary, the findings from this research provide a robust basis for the further application of algal extracts across diverse fields.

Data Availability statement

The data used in this study is available upon request from the author.

Conflict of interest

The authors declare that there is no conflicts of interest.

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