https://doi.org/10.17221/129/2025-CJFS

Microwave-assisted extraction of annatto: Effects of pH and time on antibacterial activity

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Citation: Handayani I., Septiana A.T., Sustriawan B., Karseno (2026): Microwave-assisted extraction of annatto: Effects of pH and time on antibacterial activity. Czech J. Food Sci., 44: 150–156.

Abstract: Annatto seeds possess antimicrobial properties, but extraction methods may degrade bioactive compounds. This study investigated the impact of pH-adjusted microwave-assisted extraction using distilled water on antibacterial activity to obtain safe food-grade extracts. Extractions were performed at pH values of 4, 7, and 9 for extraction times of 2, 4, and 6 min, using a microwave power setting of 100 W. The antimicrobial activity of the extracts was assessed against *Escherichia coli* and *Staphylococcus aureus* through inhibition zone analysis and viable bacterial cell count reduction. In parallel, the total phenolic content of each extract was quantified to examine the correlations between phenolic concentration and antibacterial activity. The findings revealed that extraction at pH 4 for 2 min yielded the most pronounced antibacterial activity with a minimum inhibitory concentration (MIC) of 5%. The application of a 20% annatto extract resulted in the lowest viable cell counts of both *E. coli* and *S. aureus*. Variations in pH and extraction time did not result in significant differences in total phenolic content, suggesting that antimicrobial efficacy may be influenced not only by phenolic concentration but also by the specific profile of active compounds.

Keywords: *Bixa orellana* seeds; food-grade extract; phenolic compounds; *Escherichia coli*; *Staphylococcus aureus*

The *Bixa orellana* plant (leaves, fruits, seeds, roots, stems) contains various phytochemical components with antimicrobial properties. A wide variety of phytochemicals, including carotenoids, sterols, aliphatic compounds, monoterpenes and sesquiterpenes, as well as flavonoids, polyphenols, tannins, quinones, terpenoids, alkaloids, and triterpenoids, have been reported to exhibit antibacterial, antifungal, anti-inflammatory, antioxidant, gastrointestinal motility-enhancing, haemostatic, anticonvulsant, antidiarrheal, analgesic, and diuretics properties (dos Santos et al. 2022). Annatto contains carotenoids approved by the FDA as safe natural dyes, primarily bixin and norbixin. Bixin is the major pigment, accounting for over 80% of total seed carotenoids (Carvalho et al. 2024), and is fat-soluble, whereas norbixin is water-soluble under alkaline conditions; their concentrations, including norbixinate, vary with seed maturity. Both 9'-*cis*- and *trans*-norbixin contribute to antimicrobial activity against *Staphylococcus aureus*, while annatto extracts exhibit broad antimicrobial effects against fungi and Gram-positive and Gram-negative bacteria, particularly Gram-negative species, attributed to bixin and polyphenolic compounds such as catechin, chlorogenic acid, chrysin, butein, hypolaetin, licochalcone A, and xanthohumol (Karmakar et al. 2018; Quiroz et al. 2019; dos Santos et al. 2022). Due to their effectiveness, low toxicity, compatibility with biological systems, and cost-effectiveness, annatto extracts show considerable potential as antimicrobial agents (Aluko et al. 2024).

Supported by the Jenderal Soedirman University through the 2024 Riser Dasar (Basic Research Program), Project No. 26.441/UN23.35.5/PT.01/II/2024.

https://doi.org/10.17221/129/2025-CJFS

Various extraction techniques have been employed to isolate bioactive compounds from annatto. Methods include water soaking, reflux, and Soxhlet extraction with ethyl acetate (Sabuz et al. 2020), as well as extraction using water, aqueous sodium solutions, and chloroform (Franklin et al. 2023), whereas acetone has been reported as an effective alternative (Yolmeh et al. 2025).

An aqueous extract prepared through overnight soaking demonstrated inhibitory effects against *Escheria coli* and *S. aureus* (Franklin et al. 2023). A chloroform extract of annatto inhibited the growth of *Bacillus cereus, E. coli, S. aureus, Salmonella enteritidis, Listeria innocua, Bacillus subtilis, Steptococcus pyogenes*, and *Enterococcus faecalis* (Yolmeh et al. 2025). The ethanolic extract of annatto (5 mg·disc^{-1}) exhibited potent antimicrobial activity, as evidenced by inhibition zones ranging from 13 to 25 mm. The antimicrobial activity of the extract is influenced by the pH of the distilled water used during extraction; notably, extraction at pH 4 results in greater inhibition of both *E. coli* and *S. aureus* (Handayani et al. 2021). Additionally, the extraction method plays a crucial role in determining the functional properties of the annatto extract (Quiroz et al. 2019).

Organic solvents like acetone, chloroform, and hexane are unsuitable for food applications due to the risk of residual toxicity. Water-based extraction, particularly using distilled water, presents a safer, more economical, and accessible alternative. Due to its safety and cost-effectiveness, distilled water has been widely selected as the extraction solvent for phenolic compounds from peanut meal (Akl et al. 2019). Sabuz et al. (2020) reported that combining distilled water with potassium hydroxide facilitates the conversion of bixin into a water-soluble pigment, thereby enhancing its functionality in aqueous formulations.

A major limitation of high-temperature extraction is the thermal degradation of annatto phenolic compounds, which are sensitive to light, temperature, pH, oxygen, soluble solids, and solvent type. Phenolics from *B. orellana* follow first-order degradation kinetics, with rates strongly accelerated by increasing temperature and modulated by pH and soluble solids concentration (Zapata et al. 2022).

Maceration, leaching, extraction with supercritical fluids (CO_2), dispersive liquid–liquid extraction, sonication, enzymatic extraction, microextraction, and microwave-assisted extraction (MAE) are among the most widely used methods for extracting bioactive molecules from plants. However, the extraction technique significantly influences the functional properties of the extract, consequently, conventional approaches often require long processing times, exposing active compounds to degradation and resulting in low efficiency. On the other hand, some emerging extraction techniques, such as supercritical CO_2 extraction, are costly and involve high levels of energy consumption (Afraz et al. 2023). Enhancing the stability of annatto pigments, therefore, requires minimising thermal exposure, particularly by shortening extraction times. MAE has thus emerged as a promising technique in this regard.

MAE employs microwave radiation to rapidly and uniformly heat the solvent, thereby significantly accelerating mass transfer from the solid matrix to the solvent phase. This mechanism facilitates efficient extraction of bioactive compounds, including thermolabile pigments, while preserving their integrity (Quiroz et al. 2019). Furthermore, MAE has been shown to increase crude extract yields and reduce solvent consumption when compared with conventional methods, making it a sustainable and effective option for natural pigment recovery. The antimicrobial activity of annatto extract obtained through MAE has been reported to be higher than that obtained using the leaching method (Quiroz et al. 2019).

Previous studies have investigated the colour and antibacterial properties of annatto extract produced using distilled water at varying pH levels and extraction times via the maceration method (Handayani et al. 2021). However, the potential of microwave-assisted extraction (MAE) to enhance the bioactive components of annatto extract has not yet been explored. Research into natural substances with antimicrobial activity supports the advancement of affordable and innovative technologies to improve the safety and quality of both food and pharmaceutical products. Variations in the antimicrobial efficacy of annatto extract may be influenced by factors such as extract concentration, solvent type, and the physical form of the extract (dos Santos et al. 2022).

These studies have focused on annatto extraction and the effect of distilled-water pH on antibacterial activity. As annatto bioactive compounds are concentrated in the seed outer layer, maceration can facilitate their release (Handayani et al. 2021). However, MAE with controlled extraction time and solvent pH is expected to enhance antibacterial activity compared with conventional maceration. The combined maceration MAE approach using distilled water with pH adjustment using citric acid or calcium hydroxide offers a simple,

https://doi.org/10.17221/129/2025-CJFS

food-grade method applicable to both household and industrial settings.

MATERIAL AND METHODS

Raw material. The primary material used in this study was *Bixa orellana* seeds, which were sourced from local farmers in Ledug Village, Kembaran Subdistrict, Banyumas Regency, Central Java Province, Indonesia. Other materials included citric acid (Merck, Germany). calcium hydroxide [$Ca(OH)_2$, Merck], Folin-Ciocalteau (Merck), and sodium carbonate (Na_2CO_3, Merck). The equipment used in this study included a microwave oven (Sharp R751GX, Japan), a UV–VIS spectrophotometer (Shimadzu 2UV-1800, Japan), a laminar air flow (Tryte Technologies AF-500W, China), an incubator (Memmert, Germany), an autoclave (Gemmy SA-232, Taiwan), a pH meter (Hanna Instrument, Italy), and a magnetic stirrer (Capp 15Rondo CRS-22H, Germany).

Preparation of annatto extract. Annatto extraction was performed according to Handayani et al. (2021) using 25 g of annatto seeds and 90 mL of distilled water. Maceration with a magnetic stirrer was conducted to remove the pigment layer, followed by extraction at 80 °C for 5 min. Distilled water at pH 4, 7, and 9 adjusted with 0.2 M citric acid or 0.2 M calcium hydroxide $Ca(OH)_2$ was used as the solvent. The mixture was then subjected to microwave-assisted extraction at 100 W for 2, 4, or 6 min, filtered through Whatman No. 1 paper, and the filtrate was used for subsequent analyses.

Antibacterial activity of annatto extracts analysis. The antibacterial activity of annatto extracts was assessed against *Escherichia coli* FNCC-19 and *Staphylococcus aureus* FNCC-15. The strains used in this study were obtained from the Food and Nutrition Culture Collection (FNCC), Gadjah Mada University, Indonesia. The bacterial stock cultures were initially propagated on nutrient agar (NA), then activated in nutrient broth at 37 °C for 24 h. The activated cultures were then stored at 4 °C until further experimentation. Antibacterial activity was evaluated using the agar well diffusion method. A 1% bacterial suspension of *E. coli* and *S. aureus* was inoculated separately into NA using the pour plate method. The NA was allowed to solidify in the refrigerator at 7 °C for 2 h. Wells (10 mm in diameter) were made in each of these plates using a sterile cork borer. A volume of 100 μL of annatto extract was carefully dispensed into each well using a sterile syringe and subsequently allowed to diffuse under ambient conditions for 2 h. Control experiments were set up using inocula without plant extract. The plates were incubated at 37 °C for 5 days, and the diameter of the inhibition zones (mm) was measured (Handayani et al. 2021; Irshad et al. 2025).

Determination of minimum inhibitory concentration (MIC). The MIC of annatto extract was defined as the lowest concentration inhibiting bacterial growth. Viable cell counts were used instead of optical density measurements due to colour interference from the extract (Hossain 2024). Annatto extract concentrations ranged from 5% to 35% (v/v) at 5% intervals and were prepared in nutrient broth containing bacterial suspensions (~10^7 CFU·mL^{-1}). Samples were incubated at 37 °C for 48 h, and viable cells were enumerated by the pour plate method on NA, with results expressed as log CFU·mL^{-1}. The bacterial counts from treatments containing annatto extract were compared with the control (NB without extract). The MIC was defined based on this comparison, and the concentration yielding the lowest viable cell count was selected for further analysis.

Total phenolic content (TPC) analysis. The total phenolic content (TPC) was determined using the Folin–Ciocalteu method. Gallic acid was used as the standard, with a concentration range of 5 to 125 ppm. Absorbance was measured at 765 nm using a UV–VIS spectrophotometer. For sample preparation, 100–150 mg of extract was weighed and mixed with 0.5 mL methanol, 2.5 mL distilled water, and 2.5 mL of 50% Folin–Ciocalteu reagent. The mixture was allowed to stand for 5 min, and then 3 mL of 7.5% sodium carbonate (Na_2CO_3) was added. The solution was vortexed and incubated at 45 °C for 15 min. Absorbance was then recorded at 765 nm using a UV–VIS spectrophotometer. The phenolic content was expressed as milligrams of gallic acid equivalents per millilitre of extract (mg GAE·mL^{-1}) (Fleck et al. 2023).

Statistical analysis. Statistical data were analysed using analysis of variance (ANOVA). Significant differences between means were determined using the Duncan multiple range test (DMRT) at a significance level of $P < 0.05$. Values are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Evaluation of antibacterial activity through clear zone analysis. Annatto extracts derived under varying solvent pH conditions using distilled water and extraction durations via MAE consistently exhibited inhibitory effects against *E. coli* and *S. aureus* across all treatments (Table 1).

https://doi.org/10.17221/129/2025-CJFS

Table 1. Inhibition zone diameters of *Escheria coli* and *Staphylococcus aureus* treated with annatto extracts (mean ± SD)

pH of aquadest	MAE Extraction (mins)	E. coli (mm)	*S. aureus* (mm)
4	2	1.78 ± 0.55[b]	7.58 ± 1.47[b]
	4	1.30 ± 1.08[ab]	6.99 ± 1.86[b]
	6	1.48 ± 0.48[ab]	5.67 ± 0.92[b]
7	2	1.22 ± 0.32[ab]	5.05 ± 2.79[b]
	4	1.11 ± 0.51[ab]	3.11 ± 0.36[ab]
	6	1.27 ± 0.57[ab]	2.01 ± 1.43[a]
9	2	0.91 ± 0.33[ab]	1.90 ± 1.12[a]
	4	0.83 ± 0.28[a]	1.86 ± 1.07[a]
	6	1.26 ± 0.39[ab]	2.03 ± 1.00[a]

[a,b]different letters within columns indicate statistically significant differences ($P < 0.05$)

MAE – microwave-assisted extraction

The combined effect of distilled water pH and extraction duration using the MAE technique had a significant influence on the inhibition zones of *E. coli* and *S. aureus*, as assessed by the agar diffusion assay. Among all tested conditions, extraction with distilled water at pH 4 for 2 min yielded the most pronounced antibacterial activity, as indicated by the largest inhibition zones against *S. aureus*. The antimicrobial activity of the extract is influenced by the pH of the distilled water used during extraction; notably, extraction at pH 4 resulted in greater inhibition of *S. aureus* (Handayani et al. 2021).

The higher inhibitory activity observed at pH 4 for 2 min is presumably associated with the greater solubility of phenolic compounds under acidic conditions, as well as a lower degree of degradation resulting from the shorter extraction duration. Phenolic compounds have been shown to exhibit greater solubility and stability in acidic environments (Shi et al. 2022),whereas prolonged extraction durations increase the likelihood of phenolic degradation (Antony and Farid 2022). The total phenolic content, flavonoids, and flavonols in the aqueous extract of *Matricaria pubescens* leaves were highest at pH 5, but decreased gradually with increasing pH (6 and 7). Moreover, longer treatment durations led to higher temperatures reached in the system, causing solvent volatilisation along with partial degradation of polyphenol compounds (Quiroz et al. 2019).

The application of extraction solvents at pH levels of 7 and 9 did not result in statistically significant differences in antimicrobial activity. This finding suggests that aqueous extracts prepared at these pH levels did not sufficiently enhance the solubility of bioactive compounds with potential antimicrobial properties against *E. coli* and *S. aureus*. Similarly, Quiroz et al. (2019) reported that pH had no significant linear effect on the extraction efficiency of polyphenolic compounds, leading to comparable inhibitory effects against *B. cereus* and *S. aureus*.

The antibacterial effectiveness of annatto extract against *E. coli* and *S. aureus* indicates that acidic aqueous extracts (pH 4) exhibit significantly stronger inhibition of *S. aureus* compared to *E. coli*, as evidenced by larger clear-zone diameters. However, as the pH increases to neutral (pH 7) or alkaline conditions (pH 9), the inhibitory effect is markedly reduced, with no statistically significant difference observed in the responses of *E. coli* and *S. aureus*. This disparity likely arises from the diverse phytochemicals present in annatto, such as carotenoids, phenolics, tannins, flavonoids, and alkaloids, which interact differently with Gram-positive and Gram-negative bacteria. Gram-positive bacteria (e.g. *S. aureus*) possess a thicker peptidoglycan layer and lack an outer membrane. In contrast, Gram-negative bacteria such as *E. coli* possess an outer lipid membrane that serves as a barrier, thereby limiting the penetration of phytochemical compounds. As a result, sensitivity to annatto's active compounds varies based on the bacterial cell wall.

In Gram-negative bacteria, the cell wall is more complex due to the presence of an outer lipopolysaccharide (LPS) layer located external to the peptidoglycan layer. This LPS layer often acts as a barrier, reducing the permeability of various antimicrobial compounds. In contrast, Gram-positive bacteria possess a single, thick layer of peptidoglycan, which tends to be more permeable to certain substances (Ahmed et al. 2020). The outer membrane structure of Gram-negative bacteria, particularly the presence of lipopolysaccharides (LPS), can hinder the penetration of antibacterial compounds such as carotenoids and phenolics derived from annatto extracts. Annatto is known to contain a wide array of phytochemicals, including carotenoids (bixin and norbixin), phenolic compounds, tannins, flavonoids, and alkaloids. These bioactive constituents generally exhibit stronger antibacterial activity against Gram-positive bacteria than against Gram-negative strains. These findings are consistent with those reported by Franklin et al. (2023), who demonstrated that aqueous annatto extract exhibited significant antibacterial activity, particularly against *S. aureus*.

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The determination of MIC was conducted using viable cell count analysis. Different concentrations of annatto extract significantly affected the growth of *E. coli* and *S. aureus* (Table 2).

The addition of annatto extract was generally effective in inhibiting the growth of both *E. coli* and *S. aureus*, as indicated by lower viable cell counts in the treatment groups compared with the control. This antimicrobial activity is likely attributed to the presence of bioactive compounds in annatto, particularly bixin and norbixin (carotenoids), as well as phenolic compounds, flavonoids, and tannins, which have been reported to exert antimicrobial effects through bacterial cell membrane disruption, enzyme inhibition, and oxidative stress induction (Aluko et al. 2024; Oliveira et al. 2025)

However, an unexpected increase in *E. coli* growth was observed at higher extract concentrations (30% and 35%), with cell counts exceeding those in the control. This phenomenon may be attributed to several possible factors. First, at higher concentrations, certain plant extracts can paradoxically exhibit pro-oxidant effects or provide nutrient-like components that may support bacterial metabolism rather than inhibit it (Piekarska-Radzik and Klewicka 2021). Additionally, the complex composition of annatto extract, especially in crude or semi-purified form, may contain residual sugars or nitrogenous compounds that unintentionally promote bacterial proliferation under specific conditions (dos Santos et al. 2022). Another possibility is that the high concentration of extract components may induce stress adaptation mechanisms in *E. coli*, such as upregulation of efflux pumps, repair systems, or biofilm formation, allowing the bacteria to better tolerate or even thrive in the presence of certain phytochemicals (Buchmann et al. 2023; Sui et al. 2025). This behaviour highlights the need for further characterisation of the extract composition at different concentrations and the specific response mechanisms of *E. coli* under such treatments.

Total phenol content of annatto extracts. Polyphenol compounds feature an aromatic ring structure linked to hydroxyl groups or other planar rings. These molecules exhibit mild acidity, with their solubility and bioactivity reaching optimal levels at neutral or mildly acidic pH conditions (Quiroz et al. 2019). Phenolic compounds have been widely recognised for their broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative pathogens (Ecevit et al. 2022). The total phenolic content of annatto extract obtained under different pH conditions and extraction times is presented in Figure 1.

The extraction of phenolic compounds using distilled water at pH values of 4, 7, and 9 showed no statistically significant variation in total phenolic content. As weak acids, phenolic compounds exhibit limited solubility in water, which is strongly influenced by the pH of the extraction medium. Nevertheless, despite their low aqueous solubility, phenolics extracted using water-based solvents have exhibited remarkable antibacterial properties. Notably, aqueous extracts from jaboticaba (*Plinia cauliflora*) peel rich in polyphenols demonstrated potent antimicrobial activity against *S. aureus* and *E. coli* (Fleck et al. 2023).

Although extraction at pH 4 for 6 min tended to yield higher phenolic content, the increase was not statistically significant. This trend can be attributed to the extended extraction time, which facilitates the deprotonation of hydroxyl groups in annatto-derived phe-

Table 2. Cell counts of *Escheria coli* and *Stypholococcus aureus* under varying concentrations of annatto extract at pH 4 with a 2-min extraction time (mean ± SD)

Extract concentrations (%)	Bacteria types			
	E.coli (log cfu/mL^{-1})	Δ cell *E. coli*	*S. aureus* (log cfu/mL^{-1})	Δ cell *S. aureus*
5	6.85 ± 0.06^{b}	0.38	7.25 ± 0.01^{d}	0.21
10	6.99 ± 0.12^{c}	0.24	7.24 ± 0.01^{d}	0.22
15	6.92 ± 0.03^{bc}	0.31	7.14 ± 0.01^{c}	0.32
20	6.42 ± 0.04^{a}	0.81	7.10 ± 0.01^{b}	0.36
25	6.90 ± 0.04^{bc}	0.33	7.09 ± 0.01^{b}	0.37
30	7.34 ± 0.02^{d}	–0.11	7.09 ± 0.01^{b}	0.37
35	7.30 ± 0.12^{d}	–0.07	7.00 ± 0.02^{a}	0.46
K–	7.23 ± 0.01^{d}	0.00	7.46 ± 0.01^{e}	0.00

[a–d]different letters within columns indicate statistically significant differences ($P < 0.05$)

Δ cell – the difference in cell count between the treatment (annatto extract addition); K– the control (without annatto extract), a negative Δ cell indicates that the cell count in the treatment group was higher than that in the control

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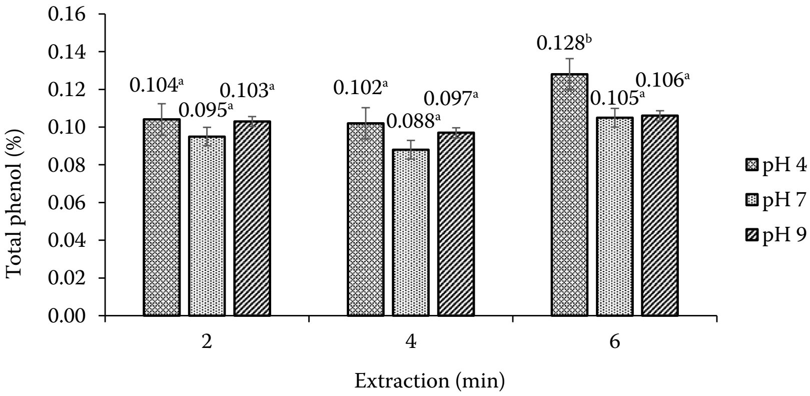

Figure 1. The total phenolic content of annatto extract obtained under different pH conditions and extraction times
Different letters mean a significant difference ($P < 0.05$)

nolics, thereby improving their solubility in aqueous media. Under alkaline conditions, phenolic -OH groups are converted into phenolate ions (O^-), which are more water-soluble in high-pH environments (Fleck et al. 2023). However, this enhanced solubility also increases the susceptibility of these compounds to oxidative degradation and may promote the formation of more complex oxidation products.

Alkaline solvents can improve the solubility of certain compounds; however, they may also induce degradation of phenolic molecules that are unstable at high pH. Polyphenols undergo degradation, dimerization, and oxidation when exposed to alkaline environments (Xiao 2022). The effects of solvent pH and extraction time in the MAE process are primarily attributed to: (*i*) enhanced mass transfer capacity and/or the system's extraction efficiency, (*ii*) the solvent's affinity for bioactive compounds, and (*iii*) the impact of microwave-generated heat on the system's micro-domains (Quiroz et al. 2019). Prolonged extraction time leads to higher system temperatures, which may result in solvent volatilization and partial degradation of polyphenolic compounds.

The antibacterial activity of annatto extract is attributed not only to its phenolic constituents but also to the presence of bixin and norbixin. Extracts produced via MAE using distilled water at pH 4 for extraction times of 2, 4, and 6 min yielded bixin concentrations of 0.59, 0.54, and 0.76%, respectively, and norbixin concentrations of 0.46, 0.27, and 0.35%, respectively (Handayani et al. 2025). Both bixin and norbixin have been documented to possess antimicrobial properties (Quiroz et al. 2019; dos Santos et al. 2022). The aqueous annatto extract obtained at pH 4 with a 2-min extraction time, which demonstrated the strongest antibacterial potential, was further evaluated to determine its MIC against the target bacteria and the concentration that resulted in the lowest viable cell count.

These findings suggest the need for further studies to optimise extract concentration and to isolate the compounds responsible for the observed stimulatory activity, in order to better understand their effects on different bacterial strains.

CONCLUSION

Microwave-assisted extraction (MAE) using distilled water and food-grade acidulants is effective in producing antibacterial extracts from *Bixa orellana* seeds. The highest antimicrobial activity was observed at pH 4 with a 2-min extraction time, under which the extract achieved a minimum inhibitory concentration (MIC) of 5% against *E. coli* FNCC-19 and *S. aureus* FNCC-15. Although no significant differences were observed in total phenolic content across the extraction conditions, the enhanced activity suggests that specific bioactive profiles and/or compound bioavailability may play a more decisive role.

Acknowledgement. The authors gratefully acknowledge the financial support from Jenderal Soedirman University through the 2024 Riset Dasar (Basic Research Program), Project No. 26.441/UN23.35.5/PT.01/II/2024, and thank Naydina Rahmawati and Inas Safira Afianita, undergraduate students involved in this research, for their valuable assistance during the study.

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Received: August 6, 2025
Accepted: February 13, 2026
Published online: April 27, 2026