EFFECT OF MICROWAVE-ASSISTED EXTRACTION TIME ON THE COLOR AND ANTI-YEAST ACTIVITY OF ANNATTO EXTRACTS FOLLOWING MACERATION

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Abstract. Annatto is a natural pigment with known antimicrobial properties. This study investigates the color characteristics and anti-yeast activity of annatto extracts produced via maceration followed by microwave-assisted extraction (MAE) at varying MAE times. Maceration was performed at 70°C for 5 minutes. The subsequent MAE was performed at a power of 100 W for 1 to 5 minutes, with 0.5-minute intervals. The anti-yeast activity was evaluated against *Candida albicans*. Color was measured using a colorimeter to determine L (lightness), a* (redness), and b* (yellowness). Anti-yeast activity was assessed by measuring the clear zone, the minimum inhibitory concentration (MIC), and the total cell count. The results show that increasing the MAE duration led to a higher level of redness but a decrease in both lightness and yellowness. A 2-minute MAE duration was found to be optimal, yielding an extract with an L value of 36.42, an a* value of 16.28, and a b* value of 13.58. The highest clear zone diameter was 6.05 mm (classified as moderate inhibition). The MIC was achieved at a concentration of 5%, with a ΔOD value of 0.114 and a colony count of 7.141 log

Keywords: Annatto, Anti-yeast activity, color characteristics, extraction time, microwave-assisted extraction (MAE)

1. Introduction

Bixa orellana is a plant belonging to the Bixaceae family, known by various names in different languages, including annatto in English, achiote in Spanish, and urucum in Portuguese. It's frequently used in the food industry as a natural colorant. The key pigments in annatto are the carotenoids, specifically bixin and norbixin. The color of the pigment, which range from yellow to red, depends on the concentration of these compounds in the seed's outer coat. Bixin is an oil-soluble pigment, while norbixin is its water-soluble derivative. Both are found in the outer coating of the annatto seed and are responsible for its distinctive color [1]

An annatto extract prepared with deionized water at temperatures between 70°C and 90°C was found to possess antibacterial properties against *E. coli* and *S. aureus*. This antimicrobial effect is attributed to the presence of key phytochemicals, specifically alkaloids, phenols, tannins, and saponins [2]. The ethanolic extract of *Bixa orellana* has also been shown to inhibit the growth of *Candida albicans* [3].

Candida spp. are significant yeast pathogens that have been isolated from various food products, including cheese [4], chicken meat [5] and mutton [6]. Contamination of meat products with *Candida albicans* can occur at multiple points along the supply chain, including during slaughtering, storage, and transportation. Consuming this contaminated meat poses a risk of *C. albicans* infection [6]. The inhibitory activity against *C. albicans* is attributed to the bioactive components present in the annatto extract 3.

Alkaloids are a structurally diverse group of compounds known for their antimicrobial activity, which operates through various mechanisms. These mechanisms are often specific to the alkaloid's class. For example, the phenanthroindolizidine alkaloids pergularinine and tylophorinidine function by inhibiting dihydrofolate reductase, thereby disrupting nucleic acid synthesis and cell division. Alkyl methyl quinolone alkaloids, on the other hand, exhibit potent antibacterial effects primarily by inhibiting bacterial respiration and key enzymes. Furthermore, the polyamine alkaloid squalamine employs a detergent-like action to disrupt the outer membrane of Gram-negative bacteria, while depolarizing and ultimately disrupting the membranes of Gram-positive bacteria [7].

Phenolic compounds exhibit diverse antimicrobial activities that are contingent on their molecular structure and the surrounding environment. Their mechanisms of action include a direct assault on the cell membrane, causing damage or structural changes. Furthermore, these compounds can reduce the expression of genes involved in quorum sensing, hindering the production of critical signaling proteins. They also interfere directly with DNA, RNA, and other proteins vital for cell function. By chelating metal ions, which are essential cofactors for bacterial proteins and membrane depolarization, phenolics can disrupt the productivity of ATP synthase. This multifaceted attack on cellular metabolic pathways ultimately compromises cell efficiency and leads to bacterial demise [8]. Tannins act as antimicrobial agents through several mechanisms: disrupting peptidoglycan formation, chelating iron ions, damaging cell membranes, and inhibiting efflux pumps [9]. Saponins act as antimicrobial agents by disrupting the lipid membranes of bacteria and fungi. They achieve this by interacting with sterols, such as cholesterol, leading to pore formation and cell lysis [10].

Antimicrobial compounds present in annatto seeds (*Bixa orellana*) can be extracted using either conventional or modern techniques. The conventional methods, such as maceration, percolation, and Soxhlet extraction, are well-established but often require longer processing times and larger solvent volumes. In contrast, modern extraction techniques offer several advantages, including shorter extraction times, higher extraction efficiency, increased crude extract yield, and reduced solvent consumption [11].

Several parameters influence the extraction rate, including sample preparation, extraction duration, sample size, extraction temperature, and solvent type [12]. The pigments and bioactive compounds in annatto seeds are primarily located in the outer seed coat and can be effectively extracted through maceration assisted by a magnetic stirrer [13]. Extraction time plays a critical role in determining the yield of bioactive compounds. Excessively long extraction may promote compound degradation through oxidation [14], while insufficient duration can result in incomplete extraction and lower yields [15]. An optimal extraction time ensures the maximum recovery of bioactive constituents, which are essential for antimicrobial efficacy. However, prolonged extraction can lead to the breakdown of compounds, diminishing the extract's biological activity. For *Cosmos caudatus* (kenikir) leaves, the optimal extraction time using [14]. Microwave-Assisted Extraction (MAE) was three minutes among the tested durations of two, three, and four minutes, resulting in the highest total phenolic content and antimicrob ial activity [16].

The extraction process, which combined maceration and Microwave-Assisted Extraction (MAE), was employed to maximize the recovery of bioactive compounds from annatto seeds. Maceration facilitated the release of pigments and bioactives from the seed coat, while MAE enhanced extraction efficiency within a shorter duration. This study investigates the effect of MAE extraction time on the color characteristics and anti-yeast activity against *Candida albicans* of the annatto extract obtained through the combined maceration–MAE method.

2. Methods

2.1. Extraction of Annatto Seeds

Maceration extraction of annatto seeds was conducted following the procedures of [17] and [18]. A total of 25 g of annatto seeds was immersed in 90 mL of solvent and stirred magnetically under mild heating for 5 minutes. Distilled water adjusted to pH 4 using citric acid served as the extraction solvent. Subsequently, Microwave-Assisted Extraction (MAE) was performed according to the method of [19], using a microwave power of 100 W for durations ranging from 1 to 5 minutes at 0.5-minute intervals. The obtained extract was then filtered through filter paper to separate the liquid extract from the seed residue.

2.2. Color Measurement of Annatto Extract

The color of the annatto extract was measured using a colorimeter based on the CIELAB color system. The instrument analyzes color through three parameters: L* (lightness), a* (red-green axis), and b* (yellow-blue axis), each typically ranging from 0 to 100. The colorimeter was calibrated and set to record L*, a*, and b* values, representing the brightness and chromatic characteristics of the extract [20].

The *L* value represents lightness, ranging from 0 (black) to 100 (white), with higher values indicating brighter samples and lower values denoting darker or more saturated colors. The *a* value quantifies chromaticity along the red–green axis, where positive values (0 to +100) indicate increasing redness and negative values (0 to –80) indicate increasing greenness. The *b* value represents chromaticity along the yellow–blue axis, where positive values (0 to +70) correspond to higher yellowness and negative values (0 to –70) correspond to higher blueness [19].

2.3. Antimicrobial Testing of Annatto Extract

The antimicrobial activity of the annatto extract was evaluated against *Candida albicans*. The antimicrobial potential of the annatto extract was assessed through the disc diffusion assay and Minimum Inhibitory Concentration (MIC) determination. The procedure began with the preparation of the microbial starter culture, followed by assessing the extract's ability to inhibit microbial growth.

A starter culture of *C. albicans* was prepared by inoculating 1 mL of the stock culture into 9 mL of Malt Extract Broth (MEB). The mixture was incubated for 24 hours, after which the cells were rejuvenated once to obtain the active microbial suspension used as the inoculum [2].

a) The disc diffusion assay

The antimicrobial activity of the annatto extract was evaluated using the disc diffusion method [21]. Sterile paper discs that had been immersed in the annatto extract for 30 minutes were removed using sterile forceps and placed onto Malt Extract Agar (MEA) plates pre-inoculated with a 1% suspension of *Candida albicans*, prepared using the pour plate technique. The plates were incubated at 37 °C for 24 hours, and antimicrobial activity was determined by measuring the diameter of the clear zone (zone of inhibition) surrounding each disc using a caliper, expressed in millimeters. A larger inhibition zone indicated stronger antimicrobial efficacy. The zones less than 5 mm indicated weak inhibition, 5–10 mm moderate inhibition, 10–20 mm strong inhibition, and greater than 20 mm pronounced inhibition [22].

b) Determination of MIC (*Minimum Inhibitory Concentration*)

The Minimum Inhibitory Concentration (MIC) represents the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism [23]. The Minimum Inhibitory Concentration (MIC) of the annatto extract against *Candida albicans* was determined according to the procedure described by [3], with slight modifications. The assay was performed using extract concentrations of 5%, 10%, 15%, 20%, and 25%. Seven sterile test tubes were prepared

for each sample and microorganism. Tube 1 served as the negative control (K−), containing only the microbial suspension in the culture medium, while Tube 2 served as the positive control (K+), containing 100% annatto extract. Tubes 3–7 were inoculated with the microbial suspension in Malt Extract Broth supplemented with annatto extract at concentrations of 5%, 10%, 15%, 20%, and 25%. All tubes were vortexed and examined visually, followed by absorbance measurement using a UV–Vis spectrophotometer at 700 nm. The samples were then incubated at 37 °C for 48 h, after which absorbance was re-measured. The lowest concentration of annatto extract yielding an OD value lower than that of the control was designated as the MIC.

3. Results And Discussion

3.1. The Color of Annatto Extract

Annatto (*Bixa orellana* L.) is widely recognized as a tropical species abundant in carotenoid pigments, particularly the nonpolar compound bixin and its polar derivative norbixin. Table 1 shows the color profile of annatto extracts produced at different MAE extraction times.

Table 1. Color measurement values of annatto extract at different MAE extraction times

Extraction time (min)	L* (lightness)	a* (redness)	b* (yellowness)
1.0	37.48	14.01^{a}	13.67
1.5	36.61	15.89^{bc}	14.40
2.0	36.42	16.28^{cd}	13.58
2.5	36.38	16.35^{cd}	15.91
3.0	36.69	16.71^{cd}	13.68
3.5	36.57	14.26^{ab}	15.59
4.0	36.34	15.44^{abc}	15.20
4.5	35.54	20.45^{e}	11.96
5.0	35.68	18.04^{d}	14.16

Note: Values followed by different superscript letters within the same column indicate significant differences ($P < 0.05$).

Extraction time had a pronounced effect on the intensity of redness in the annatto extract. As shown in Table 1, the highest a* value (20.45), representing the degree of redness, was obtained at a microwave-assisted extraction (MAE) time of 4.5 minutes. In contrast, the lowest value (14.01) was observed at 1 minute. The progressive increase in a* values from 1 to 3 minutes indicates efficient bixin extraction within this range, as microwaves rapidly heat intracellular water molecules, disrupt seed cell walls, and enhance pigment diffusion into the solvent [23]. A slight decline in redness at 3.5 minutes (a* = 14.26) suggests the onset of pigment degradation, possibly due to uneven energy distribution during extraction. The redness intensity then increased again, peaking at 4.5 minutes, indicating that this duration provided optimal thermal energy for releasing bixin and norbixin pigments from the seed matrix. Annatto seeds are rich in these carotenoids, which impart yellow to deep-red hues [24]. At 5 minutes, a decrease in redness intensity was observed, likely resulting from heat accumulation or superheating that promotes pigment degradation [4]. Prolonged microwave exposure can also accelerate the thermal breakdown of bixin and other bioactive compounds [23]. Variations in extraction time did not significantly affect the lightness and yellowness of the resulting annatto extract. Both color parameters tended to decrease at an extraction time of 4.5 minutes. The notable reduction in yellowness at this duration may be attributed to the degradation of the main pigments, bixin and norbixin, which reduced the color stability of the extract [25]. Prolonged extraction of annatto seeds accelerates norbixin degradation [5]. This finding aligns with the results of [26], who observed that the yellowness intensity of annatto extracts obtained through

a combination of maceration and microwave-assisted extraction (MAE) remained statistically unchanged even with prolonged extraction times.

3.2. Antimicrobial Activity of Annatto Extract Against *Candida albicans*

The antimicrobial activity of annatto extract against *Candida albicans* in this study was assessed through clear zone measurement using the disc diffusion method, minimum inhibitory concentration (MIC), and total plate count (TPC) assays.

3.3. Clear Zone (Inhibition Zone)

The antimicrobial activity of the annatto extract against *C. albicans* was assessed by the disc diffusion method. Clear zones around the discs indicated microbial inhibition [27] where larger diameters signified greater potency. Inhibition zone diameters varied with extraction time, as shown in Table 2 for extracts obtained by combined maceration and microwave-assisted extraction (MAE).

Table 2. Inhibition zone diameter of annatto extract against *C. albicans* at different MAE extraction times

Extraction time (min)	Inhibition zone (mm)
1.0	1.09^{a}
1.5	1.66^{b}
2.0	6.05^{f}
2.5	1.18^{a}
3.0	2.14^{c}
3.5	2.60^{d}
4.0	1.50^{ab}
4.5	3.79^{e}
5.0	1.27^{ab}

Note: Values followed by different superscript letters indicate significant differences ($P < 0.05$).

As shown in Table 2, the inhibition zone diameters of the annatto extract against *C. albicans* varied significantly with different MAE extraction times ($P < 0.05$). The largest inhibition zone (6.05 mm) was observed at an extraction time of 2 minutes, indicating moderate antifungal activity. Inhibition zones below 10 mm are generally considered weak to moderate [27]. In this study, the annatto extract exhibited moderate inhibition, which suggests partial effectiveness of the bioactive compounds, particularly bixin and norbixin, in disrupting fungal cell membranes. The moderate inhibition may be attributed to the relatively low solubility and polarity of carotenoid compounds, which can limit their diffusion through the agar medium [28]. Moreover, *C. albicans* possesses a robust cell wall composed of glucans and mannoproteins that can reduce compound permeability and decrease sensitivity to plant-derived antimicrobials [29].

3.4. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the annatto extract against *C. albicans* was determined to identify the lowest concentration capable of inhibiting visible microbial growth [22]. The MIC test provides quantitative data on the antimicrobial potential of the extract by measuring the concentration at which fungal growth is suppressed. A lower MIC value indicates stronger antimicrobial efficacy. The MIC values of annatto extract obtained under different extraction times are presented in Table 3.

Based on Table 3, the minimum inhibitory concentration (MIC) of the annatto extract against *Candida albicans* was consistently observed at a 5% concentration across all extraction times. This indicates that the extract exhibited effective antifungal activity even at relatively low concentrations. The lower ΔOD values compared to the negative control reflect a significant reduction in microbial growth, confirming the inhibitory effect of the extract.

The consistent MIC across different extraction times suggests that the key antifungal compounds were effectively extracted under all microwave-assisted extraction (MAE) durations tested. However, slight variations in ΔOD among extraction times may result from differences in the concentration or stability of bioactive constituents such as bixin, carotenoids, phenolics, and terpenoids, which are known to contribute to the antimicrobial properties of *Bixa orellana* extracts [6,7].

Table 3. Mean ΔOD values in the MIC assay of annatto extract with varying MAE extraction times against *C. albicans*

Extraction time (min)	ΔOD difference						
	Annatto extract concentration (%)						
	K-	5%	10%	15%	20%	25%	K+
1	0.144^{d}	0.104^{c}	0.090^{bc}	0.086^{bc}	0.082^{bc}	0.065^{ab}	0.052^{a}
1.5	0.144^{d}	0.172^{e}	0.139^{d}	0.086^{c}	0.077^{bc}	0.062^{b}	0.038^{a}
2.0	0.144^{e}	0.114^{d}	0.087^{c}	0.083^{c}	0.074^{bc}	0.53^{b}	0.016^{a}
2.5	0.144^{f}	0.121^{e}	0.095^{d}	0.087^{cd}	0.082^{bc}	0.073^{ab}	0.065^{a}
3.0	0.144^{d}	0.105^{c}	0.119^{c}	0.109^{c}	0.088^{b}	0.085^{b}	0.056^{a}
3.5	0.144^{e}	0.126^{d}	0.104^{c}	0.097^{c}	0.072^{b}	0.047^{a}	0.037^{a}
4.0	0.144^{e}	0.127^{d}	0.085^{c}	0.086^{c}	0.065^{b}	0.055^{b}	0.021^{a}
4.5	0.144^{f}	0.118^{e}	0.092^{d}	0.084^{cd}	0.075^{bc}	0.067^{b}	0.032^{a}
5.0	0.144^{c}	0.097^{b}	0.089^{b}	0.085^{b}	0.082^{b}	0.059^{ab}	0.042^{a}

Note: Values followed by different superscript letters within the same row indicate significant differences ($P < 0.05$).
K(–): 100% microbial suspension without extract
K(+): 100% annatto extract
ΔOD: the difference in absorbance measured at 700 nm before and after incubation.

MAE enhances mass transfer and disrupts plant cell walls, facilitating the release of these active molecules, though excessive heating or prolonged extraction may also lead to partial degradation [31]. The observed dose-dependent reduction in ΔOD is consistent with the general antimicrobial behavior of plant-derived extracts, where higher concentrations produce stronger inhibitory effects by altering cell membrane integrity, interfering with ergosterol synthesis, or disrupting metabolic pathways in fungal cells [32]. Although ΔOD measurement offers a rapid quantitative estimation of growth inhibition, it cannot distinguish between fungistatic and fungicidal effects. The ΔOD measurement offers a convenient quantitative indicator of microbial growth inhibition. Therefore, additional assays such as minimum fungicidal concentration (MFC) determination, colony-forming unit (CFU) counts, or time-kill kinetics are recommended for a more comprehensive evaluation of antifungal efficacy [33].

3.5. Total Plate Count (TPC)

The total plate count (TPC) assay was performed to quantify viable *C. albicans* cells following treatment with annatto extract. This method enables the evaluation of microbial reduction after extract exposure, where lower colony counts indicate stronger antimicrobial activity [34]. Treatment with annatto extract obtained from a 2-minute microwave-assisted extraction (MAE) and applied at a 5% concentration resulted in a microbial count of 7.141 log CFU/mL, compared with 7.304 log CFU/mL in the control medium (MEA without extract). The reduced cell count in the treated sample demonstrates that the annatto extract exerted a measurable inhibitory effect on *C. albicans* growth.

Although the reduction was modest, the findings indicate that even a low concentration (5%) of annatto extract can partially suppress microbial proliferation. This inhibitory activity is likely attributed to bioactive constituents such as bixin, norbixin, phenolic compounds, and

terpenoids, which have been reported to disrupt microbial membranes and interfere with cellular metabolism [29,30]. The short 2-minute MAE duration likely enhanced the extraction efficiency of these thermolabile compounds while minimizing degradation, thereby preserving their antimicrobial potential [31]. Previous studies have also demonstrated that *Bixa orellana* extracts possess moderate antibacterial and antifungal properties by compromising cell wall integrity and increasing membrane permeability, leading to leakage of intracellular contents [32].

4. Conclusion

Microwave-assisted extraction (MAE) time significantly influenced both the color properties and antimicrobial activity of annatto extract. The optimal extraction time of 4.5 minutes yielded the highest redness intensity (*a* value), indicating enhanced pigment release and color quality. In antimicrobial assays, the extract exhibited inhibitory effects against *Candida albicans*, as shown by the formation of clear zones, reduced ΔOD values at the MIC level, and lower total plate counts compared to the control. The extract obtained from a 2-minute MAE showed measurable antifungal activity, reducing viable cells to 7.141 log CFU/mL. These results suggest that MAE effectively enhances the extraction of bioactive and chromophoric compounds from annatto seeds, producing extracts with promising color stability and moderate antimicrobial potential. Optimizing extraction time is therefore critical to balance pigment yield, compound integrity, and biological efficacy, supporting the potential use of annatto extract as a natural colorant and antimicrobial agent in food systems.

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