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FULL PAPER

Color and antibacterial activity of annatto extracts at various pH of distilled water solvent and extraction temperature

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Abstract

Annato (*Bixa orrelana* L.) has been renowned as a tropical plant rich in carotenoid pigments such as nonpolar bixin and polar norbixin. This study was aimed to obtain natural colorant and antimicrobials from annatto extracts. The extraction was carried out by maceration for 10 mins using distilled water as the extraction solvent at various pH and extraction temperatures. The variations of solvent pH used in this research were 4, 7, and 9, while that of extraction temperatures were 70, 80, and 90°C. The potential of annatto extract as an antimicrobial agent was tested by analyzing the extract's ability to inhibit pathogens and its phytochemical compounds. *Escherichia coli* and *Staphylococcus aureus* were used as the pathogenic bacteria by using the agar diffusion method. The color of annatto extracts was measured using Munsel Chard to determine the level of hue (color), value (brightness), and chroma (intensity), as well as maximum absorbance. The results showed that all extracts have the potential to inhibit *E. coli* and *S aureus* (weak-moderate). The observed annatto extracts had different color intensities as indicated by the hue, value, and chroma and a maximum absorbance at a wavelength of 400 nm.

1. Introduction

In recent years, public concern about synthetic pigments and preservatives' safety has led to increasing interest in developing natural food colorants and preservatives from plant tissues, especially from some edible sources (He *et al*., 2015; Ramli *et al*., 2017). *Bixa orellana* (annatto) is one of the plants that have high potential as a colorant. Annatto obtained from *Bixa orellana* fruit is one of the natural pigments used as a natural food colorant. The main pigment of annatto is carotenoid composed of bixin and norbixin (Gallardo-Cabrera and Rojas-Barahona, 2015) as well as β carotene, cryptoxanthin, lutein, zeaxanthin, and methyl bixin (Scotter *et al*., 2000).

Annato pigment has a high tinctorial value and an outer colour range comprising red, orange, and yellow hues (Husa *et al*., 2018). This range of colours is an additional advantage of the annatto carotenoids over other carotenoids such as carrot and beetroot, which only show their respective colours (Silva *et al*., 2008). Bixin (nonpolar) is more soluble in vegetable oil; on the other hand, norbixin (polar) is more soluble in an aqueous solution. As a colorant, annatto is used in cheeses, sausages, meat, and candies industries (Silva *et al*., 2008). The dairy industry is the biggest usage of annatto pigment. Annato extract, apart from being a potential colorant, also has the potential to be a natural antimicrobial (Venugopalan and Giridhar, 2012; Yolmeh *et al*., 2014).

Carotenoids are susceptible to enzymatic or nonenzymatic oxidation, which depends on the carotenoid structure, oxygen availability, enzymes, metals, prooxidants and antioxidants, high temperature, and light exposure (Mezzomo and Ferreira, 2016). Colour loss of annatto extracts occurs upon prolonged exposure to light, elevated temperature, and in the presence of sulfur dioxide (Satyanarayana *et al*., 2003). Gu *et al*. (2008) evaluated the addition of HCl to optimize the extraction process. The results proved that the pre-treatment using HCl was the most effective method for carotenoids extraction.

Three main commercial processes are commonly used to extract the pigment from dried annatto seeds, direct extraction into oil, direct extraction into aqueous

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alkali, or indirect extraction with solvents (Scotter *et al*., 2000). The major colouring principles produced by direct oil extraction are 9′-*cis*-bixin, *all-trans*-bixin, to provide a colour formulation suitable for fat- or oil-based products such as margarine. Annatto extracts are susceptible to oxidative degradation.

Direct aqueous alkali extraction produces alkali metal or ammonium salt solutions of 9′-*cis*norbixin plus a small amount of the very poorly soluble all-trans isomer. Alternatively, the free acid form of norbixin can be precipitated with dilute acid, filtered, washed, and dried to produce a solid formulation (Scotter *et al*., 2000). Annatto extraction using distilled water is profitable since distilled water is an edible solvent, easy to obtain, and cheap. In this study, annatto extraction was carried out using acidic and alkaline distilled water at several different extraction temperatures. According to Satyanarayana *et al*. (2003), yellowness of extract increased, and redness decreased with an increase in temperature. However, the colour characteristics and antibacterial activity of distilled-water based extract at acidic and alkaline pH and various extraction temperatures have not been widely published.

2. Materials and methods

2.1 Annato extraction

Annato extraction was carried out in accordance with studies conducted by (Rosamah *et al*., 2009) and Abayomi *et al*. (2014). A total of 25 g of annatto seeds was added to 90 mL of distilled water as the solvent. Maceration was performed through a magnetic stirrer at various heating temperatures of 70, 80, and 90^{o}C for 30 mins. Two acidity levels of distilled water were applied, pH 4 and 9. Distilled water with pH 4 was adjusted by adding citric acid, while that with pH 9 was adjusted using $Ca(OH)_2$. After extraction, the mixture was filtered to separate the extract from the annatto seeds.

2.2 Phytochemical analysis

Preliminary screening of secondary metabolites such as alkaloids, flavonoids, saponins, coumarins, anthraquinones, terpenoids, steroids and sterols were carried out according to the common phytochemical methods described by Harborne (1973).

2.3 Antimicrobial activity

The antibacterial activity of annatto extracts was assessed against *Escherichia coli* FNCC -19 and *Staphylococcus aureus* FNCC-15. The stock bacteria were grown on nutrient agar (Merk) then activated using nutrient broth (Merk) at 37^{o}C for 24 hrs and then kept at 4^{o}C before further experiments (Bakht *et al.,* 2011). Agar well diffusion method was followed to determine the antimicrobial activity. Wells (10 mm diameter) were made in each of these plates using a sterile cork borer. Activating cultures bacteria of the pathogen were cultured in nutrient broth by poured plates. About 100 µL of annatto extracts were added using a sterile syringe into the wells and diffused at room temperature for 2 hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 5 days. The diameter of the inhibition zone (mm) was measured.

2.4 Colour extracts measurement

Colour of extracts measured utilizing Munsell Colour Chart (Ahmed *et al*., 2002) to determine hue, value, and chroma. The maximum absorbance of the extract was determined using spectrophotometry. Hue states the name of the colour, and the value represents the level of brightness or colour brilliance, while chroma declares colour intensity, strength, or purity. The maximum absorbance of the extract was determined using spectrophotometry, according to Abayomi *et al*. (2014), with some modification.

3. Results and discussion

3.1 Phytochemical compounds

The phytochemical diversity of antimicrobial compounds includes terpenoids, saponins, phenolics and phenylpropanoids, pterocarpans, stilbenes, alkaloids glucosinolates, hydrogen cyanide, indole, and also elemental sulfur, the sole inorganic compound (Shakeri *et al*., 2012). In this study, the phytochemical analysis of the annatto extract (Table 1) showed the presence of different groups of secondary metabolites such as alkaloid, phenol, tannin, and saponin

Annatto extract in distilled water solvent showed the presence of alkaloid, phenol, tannin, and saponin, but not flavonoid. The alkaloid content, tannin, and saponin in the extract were moderate to appreciable, while phenol was in trace amount, but flavonoid was not detected. The lower phenol content in extracts might be explained by the low solubility of phenols in distilled water. The extract had the highest level of alkaloid and tannin compared to other compounds. The relationships between tannins contents and extraction solvents could be related to the polymerization degree for the tannins extracted by different solvents (Naima *et al*., 2015). The interactive abilities of solvent and flavonoids or tannins compounds are probably related to chemical compositions and structures. The tannins' solubility is correlated to the degree of polymerization due to the increase in the number of hydroxyl groups –OH (Felhi *et al*., 2017). In general, phenolic compounds' solubility

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Table 1. Phytochemical screening in seed extract of annatto

Treatment		Phytochemical				
pH of solvent	Temperature (°C)	Alcoloid	Flavonoid	Phenol	Tannin	Saponin
4	70	++	-	+	+	+
	80	+++	-	+	++	++
	90	+++	-	+	+++	+++
9	70	++	-	+	++	++
	80	+++	-	+	++	++
	90	++	-	+	+++	++

+++ Appreciable amount, ++ Moderate amount, +Trace amount, -Absent

depends not only on the type of solvent used but also on the degree of polymerization and interaction of phenolics with other phytochemicals, vitamins, and minerals (Naczk and Shahidi, 2004).

The solvent's pH did not affect phytochemical levels, but increasing in temperature caused an increase in phytochemical levels. It was suspected that the phytochemical compounds in annatto have similar solubility levels in distilled water at acidic and alkaline pH. The higher the extraction temperature caused more water in the extract to evaporate, thus, the extract's phytochemical compounds concentration increased.

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve as antimicrobial. The recovery of phytochemicals from the plant could be influenced by the dielectric constant, chemical structure of organic solvents, and chemical properties of plant phytochemicals.

3.2 Antibacterial activity of extracts

Evaluation of the antibacterial activity of annatto extracts was determined by measuring the inhibition zone against *E. coli* and *S. aureus* (Table 2)

Table 2. Antimicrobial activity of annatto extract

Treatment		Inhibition zone (mm)	
pH extraction	Temperature (°C)	*E. coli*	*S. aureus*
4	70	6.67 ± 1.11^{a}	6.33 ± 2.08^{bc}
	80	7.00 ± 1.00^{a}	9.33 ± 0.57^{a}
	90	7.00 ± 1.36^{a}	7.33 ± 1.52^{ab}
9	70	3.33 ± 2.31^{ab}	5.67 ± 1.15^{bc}
	80	4.67 ± 0.60^{b}	4.33 ± 0.57^{c}
	90	7.00 ± 1.70^{a}	4.33 ± 1.15^{c}

Values with different superscript within the same column are significantly different ($p < 0.05$).

As seen in Table 2, all the extracts were potent antimicrobials against *E. coli* and *S aureus*. Annato extract produced from pH 4 at 80°C showed the highest degree of inhibition against *S aureus* with a diameter of 0.93±0.06 mm, while the lowest degree was produced from pH 9 at 70°C. Based on the inhibition zone's diameter, the annatto extract produced at pH 4 had moderate inhibition at pH 9. According to Tari and Handayani (2015), the antibacterial activity of a bacterium is determined based on the diameter of the bacterial inhibition zone against indicator bacteria, which are generally pathogenic bacteria with a range of >20 mm = very strong, 10-20 mm = strong, 5-10 mm = moderate and <5 mm = weak. The antibacterial activity is thought to be produced by phenolic compounds.

In this study, *S. aureus* was more susceptible to the tested extracts than E. coli. Indeed, the majority of the compound's extracts assayed for their antibacterial properties showed a more pronounced effect against the Gram-positive bacteria. The resistance of Gram-negative bacteria has been ascribed to their hydrophilic outer membrane, which can block the penetration of antibacterial compounds into the target cell membrane. The wall of Escherichia coli is very rich in lipopolysaccharide (LPS) that prevents antibacterial molecules such as phenol. The resistance of Staphylococcus aureus to some plant extracts can be explained by the heterogeneous wall structure of the bacteria: the presence of the exopolysaccharide containing an outer layer (glycocalyx), the presence of certain components such as the teichoic acid, and links between the various components highly cross-linked polymer give the walls an unknown tertiary structure (Bouyahya, 2016).

Phenolic compounds are one of the most diverse groups of secondary metabolites found in edible plants. It was reported that an antimicrobial action of phenolic compounds was related to the inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes. Increased membrane permeability is a major factor in the mechanism of antimicrobial action, where compounds may disrupt membranes and cause a loss of cellular integrity and eventual cell death.

Tannins bind to proline-rich proteins and interfere with protein synthesis. Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in

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response to microbial infection. It should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. The antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell (Kumaravel and Alagusundaram, 2014). Kumaravel and Alagusundaram (2014) reported that the hydroxyl (-OH) group in phenolic compounds might cause bacterial inhibition and described the importance of double bonds (number and position) concerning antimicrobial effectiveness.

Phenols interact with proteins form phenol protein complexes. The bond between protein and phenol is a weak bond and breaks down immediately. Free phenol will penetrate bacterial cells, causing precipitation and protein denaturation. Phenol can cause protein coagulation so that the cell membrane undergoes lysis. The occurrence of lysis in the cell membrane results in leakage in the cell so that the essential metabolites needed by microbes leave the cell. Phenol in the cell will damage the cell work system, damage the cytoplasmic membrane, which results in inhibition of cell growth or cell death, denatures proteins, nucleic acids, inhibits nucleic acid and protein synthesis (Ngazizah *et al*., 2017)

Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compounds indicates the mechanism in which steroids specifically associate with membrane lipid and exert their action by causing leakages from liposomes

3.3 Colour extract measurement determined using Munsell colour chard

Hue, value, and chroma were determined using the Munsell colour chart (Ruck and Brown, 2015). The results of the colour readings of annatto extract using the Munsell chart showed that the use of different pH of the solvent and temperature extraction produces different extract hue.

3.3.1 Hue

The hue of extract determination using the Munsell Chart is shown in Table 3.

Table 3 indicates that the hue of all off extracts was YR, which showed yellowish red (orange). Extraction using pH 4 of distilled water at 80^{o}C resulted in the highest hue of annatto extract. Acidic solutions at high temperatures are thought to be more capable of extracting pigment compounds to produce a higher hue (Saputri *et al*., 2017). High temperature, acidic pH solvent, and stirring using a magnetic stirrer are thought to enhancement abrasion of the annatto seed exocarp, thereby increasing colour intensity. The highest colour of the annatto extract was 7.5 YR of hue produced by extraction at pH 4 and 80^{o}C. This value was not different from that produced by extraction at pH 4 and 90^{o}C.

Increasing the extraction temperature from 70^{o}C to 80^{o}C at pH 4 increased the colour intensity of the extract indicated by the hue's enhancement. The increase in hue level indicates a higher orange (yellowish-red) colour. An increase in extraction temperature causes a change in stereoisomers from cis-bixin to trans-bixin, which is more stable (Satyanarayana *et al*., 2003). Cis bixin is most soluble in organic polar solvents, giving it an orange colour. High temperature can change cis bixin to trans bixin, which is more stable and gives a red colour. Rosamah *et al*. (2009) also reported that Annatto pigment is more stable at acidic pH. According to Scotter (2009), bixin and norbixin are relatively polar carotenoids.

Table 3. The hue of extract based on measurement using Munsell Chart

Treatment		Colour criteria
pH extraction	Temperature (^{o}C)	Hue
4	70	2.5 YR^{c}
	80	7.5 YR^{a}
	90	7.5 YR^{a}
9	70	5 YR^{b}
	80	5 YR^{b}
	90	5 YR^{b}

Hue in the same column for each test, followed by a different superscript are significantly different ($p < 0.05$). YR: Yellow-Red colour

The colour component of the extract in distilled water was norbixin in the form of cis norbixin or trans-norbixin (Satyanarayana *et al*., 2003). It is suspected that the form of norbixin in the extract is more dominant so that the resulting colour is more stable. According to Mota *et al*. (2016), bixin produces a red colour while norbixin produces an orange colour.

Ruck and Brown (2015) reported that hue is divided into five principal sections, based on the colours red (R), yellow (Y), green (G), blue (B), and purple (P). These five hues have intermediate classifications as well, YR lies between red and yellow, resulting in 10 distinct hue designations, each with four sub-divisions. Hue is the dominant colour spectrum according to its wavelength (Priandana *et al*., 2016).

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3.3.2 Value

Value is the lightness of the colour according to the amount of light reflected (Astiningrum *et al*., 2018). The value of extract annatto determination using the Munsell Chart is shown in Table 4.

Table 4. Value of extract based on measurement using Munsel Chart

Treatment		Colour criteria
pH extraction	Temperature (°C)	Value
4	70	5.5[b]
	80	6[a]
	90	6[a]
9	70	4[e]
	80	5[c]
	90	4.5[d]

Values with different superscript within the same column are significantly different ($p < 0.05$).

Based on Table 4, extraction using pH 4 distilled water produced higher value extracts, which indicates a lighter colour. The higher the value, the more light is reflected. It was suspected that pH 4 of solvents is better to extract the annatto pigment. The temperature of 80°C was more effective for extracting annatto pigments compared to temperatures of 70°C and 90°C. The more pigment produced, the more light is transmitted, resulting in a higher value.

This result is different from Paryanto *et al*. (2014), who stated that annatto extraction using 0.25 N NaOH solvent is more effective at 60°C, and extraction at 70°C produces lower amounts of bixin. Furthermore, Paryanto *et al*. (2014) stated that annatto extraction at higher temperatures and a long time could cause the bixin compound's degradation. The annatto extraction using distilled water at a temperature of 90°C produced the extract with the highest absorbance compared to extraction at lower temperatures (Rosamah *et al*., (2013).

3.3.3 Chroma

Chroma is a dimension related to the bright or gloomy colour. According to Widiantoro (2008), colour intensity is the level of strength/purity of a colour. The higher of chroma, the brighter of colour, the higher the purity of its colour. Chroma of extract determination using Munsell Chart as shown in Table 5.

Table 5 shows that distilled water pH 4 produced the highest chroma, while pH 9 produced the lowest chroma. It was suspected that pH 4 distilled water was better to be used to extract colour, resulting in more pigment. More pigment produced a brighter/stronger colour. On the other hand, distilled water pH 9 is thought to be less able to extract annatto pigment resulting in lower colour intensity (chroma).

Table 5. Chroma of extract based on measurement using Munsel Chart

Treatment		Colour criteria
pH extraction	Temperature (°C)	Chroma
4	70	7[d]
	80	10[a]
	90	10[a]
9	70	8[c]
	80	10[a]
	90	9[b]

Values with different superscript within the same column are significantly different ($p < 0.05$).

3.4 Maximum absorbance of extracts

The results of the absorbance of annatto extract at variations of solvent pH and extraction temperature are shown in Figure 1.

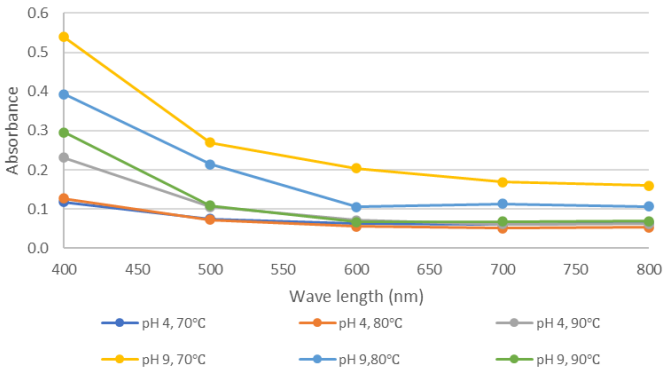

Figure 1. The absorbance of annatto extract at variations in solvent pH and extraction temperature.

The maximum wavelength of each extraction was at the same wavelength, namely 400 nm with different absorbance values. Extraction using pH 9 of distilled water at a temperature of 70°C produced the highest absorbance of 0.539. Extraction using distilled water pH 4 at 70°C produced the lowest absorbance of 0.045 (the extract was diluted 200×). Judging from the curve measured using a visible spectrophotometer, the shape of the curve is relatively the same. Extraction using pH 9 solvent resulted in a higher absorbance. It is suspected that the pH 9 extract had a compound with a higher conjugation rate, so it was better able to absorb the colour.

4. Conclusion

Annatto extraction using distilled water at a temperature of 70 to 90°C produces extracts containing phytochemicals alkaloid, phenol, tannins, and saponins, which can be antibacterial against *E. coli* and *S aureus*

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with low to moderate inhibition power. Annatto extraction using distilled water pH 4 at 80 and 90°C results in the highest extract colour, namely 7.5 YR of hue, 6 of value, and 10 of chroma. All extracts have a maximum wavelength at 400 nm, while the absorbance of the extract produced in pH 9 distilled water is higher than that produced in pH 4.

Conflict of interest

The authors declare no conflicts of interest.

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