ANTI – MICROBIAL PROPERTIES OF DYES EXTRACTED FROM HIBISCUS FLOWER (ZOBO)

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ABSTRACT

Zobo dye was successfully extracted from hibiscus flower using methanol as the extracting solvent in a soxhlet apparatus. The dye was characterized using UV-visible analysis and FTIR spectroscopy. Ethanol was used to dissolve the dye sample for the UV – visible spectra analysis which gave maximum absorption at 548nm. From the FTIR spectrum of the extracted dye, the Aromatic C = C stretching is indicated at 1521 cm^{-1}, OH band is observed at 3377cm^{-1}, C = O appeared at 1733cm^{-1}, the vibration due to C-O occurs at 1260 – 1066 cm^{-1}, and the C-H vibration may be observed at 2956 cm^{-1}. The dye extract was tested for microbial activity against *Klebsiella pneumonia* and *Staphylococcus aureus* isolates. There were inhibitory activities at all the concentration of the extract on the isolates. As expected the mean zone of inhibition of the isolates decreases with decrease in concentration of the extract. The result also suggested that the extract and the control (Ciprofloxacin) complemented each other's activity on the isolates. Thus the best result was obtained at 25 mg/ml of the dye extract when the total mean zone of inhibition of the isolates reached its peak of 22 mm and 24 mm for the *Klebsieller pneumonia* and *Staphylococcus aureus* respectively. The minimum inhibitory concentration of the dye extract against the test organisms, were found to be 6.25 mg/ml and 12.5 mg/ml while the minimum bactericidal concentration was obtained as 12.5 mg/ml and 25 mg/ml for the *Klebsiella pneumonia* and *Staphylococcus aureus* respectively.

Key words: Hibiscus flower, dyes, microbial properties

INTRODUCTION

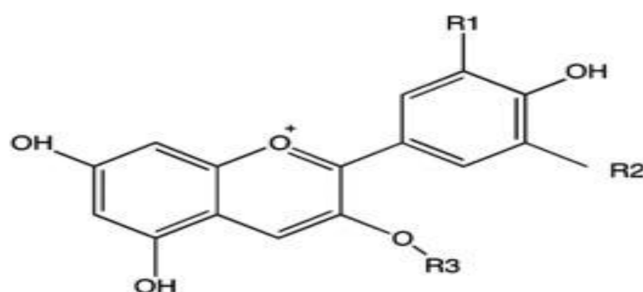
The use of synthetic dyes became dominant over the years against natural dyes due to wide availability, low cost, high range of hue (colour) and better fastness properties. However, recently, attention is going back to natural dyes due to environmental, health and safety concern.

Plant-derived natural dyes are easily extracted, completely biodegradable, and readily available. Many natural dyes, including anthocyanin, cyanine, carotene, tannin, and chlorophyll, have been studied [1].

The flowers of Hibiscus are edible and are used as salads in the Pacific Islands. The flower is additionally used in hair care as a preparation. It is also used to shine shoes in certain parts of India. It can also be used as a pH indicator. When used, the flower turns acidic solutions to a dark pink or magenta color and basic solutions to green. The flowers of the hibiscus are also a major source of natural dyes [2].

Pharmacological studies of anthocyanins in hibiscus have shown that they have antioxidant activity in patients with atherosclerosis. In several countries, it is used as a natural medicine for treating hypertension, pyrexia, liver disorder and microorganism growth limitation, as well as a digestive and sedative [3].

The anthocyanins are a group of flavonoid derivatives and natural pigments present in the dried flowers of *Hibiscussabdariffa* and their colour varies with pH.



Cyanidin-3-sambubioside (R1= OH; R2= H; R3= Sambubioside)
Delphinidin-3-sambubioside (R1= OH; R2= OH; R3= Sambubioside)
Cyanidin-3-glucoside (R1= OH; R2= H; R3= Glucose)
Delphinidin-3-glucoside (R1= OH; R2= OH; R3= Glucose)

Scheme 1: Chemical structures of main anthocyanins [4]

The studies about the effect of plant extract against different types of bacteria are still one of the most important fields of researches [5]. The extracts thus obtained after extraction may be used as medicinal agents [6] normally expected to contain phytochemicals. These chemicals are the natural defense system against diseases and pest [7].

Phytochemicals are bioactive components present in plants. They includes: tannins, alkaloids, saponins, flavonoids and steroids [8]. Fruits that are brightly coloured yellow, orange, red, green;

blue and purple generally contains the most phytochemicals and the most nutrients. Phytochemicals are natural bioactive components found in vegetables which may reduce cancer, strokes, hinder the aging process and antimicrobial properties [9]. They have complimentary and overlapping mechanisms of action in the body including antioxidant effects, modulation of detoxification and enzymes stimulation of immune system, modulation of hormone metabolism, anti-bacteria and antiviral effects [10].
In this study, dye was extracted from Hibiscus flower using standard procedure, characterized and the microbial properties investigated.

MATERIALS AND METHODS

Materials: Hibiscus flower, Acetone, Dimethyl sulphoxide (DMSO), Ethanol, Methanol, Distilled water, Cotton wool, Klebsieller pneumonia isolates, Staphylococcus aureus isolates

Equipment: Soxhlet apparatus, UV Visible Spectrophotometer (Model: Agilent Technologies, Cary Series), FTIR Machine (model: Agilent Technologies, Cary 630 FTIR), Oven (Cole Parmer, Chicago, Illinois 60648), Weighing balance, Measuring cylinder (100 ml), scissors, Syringe, Heating mantle, Flat bottom, conical flask, Swap stick and Refrigerator.

Experimental methods

Collection and preparation of sample

The hibiscus flower (dried form) was obtained from community market Ahmadu Bello, University (A.B.U), Zaria, Nigeria. The flower was separated from the seed and pounded thoroughly and sieved through 2.5 mm sieve. The powder sample (500g) was packed in a high density polyethylene (HDPE) bags and kept in air tight container for further use.

Extraction of dyes from hibiscus Sabdariffa (zobo flower) (Soxhlet extraction method)

Soxhlet apparatus was employed in the extraction of the dye using methanol as the solvent. The powdered hibiscus flower was packed into cotton bags. Each cycle used 50g of the sample and 100 ml of methanol as reagent. The methanol used was distilled (purification) using rotary evaporator at 66^oC at a speed of 25 rpm.
Cotton wool was placed in an extraction chamber, which was suspended above a flask containing methanol as a solvent at 80^oC for 1 h. The flask was heated and the solvent evaporated and moved up into the condenser where it was converted into a liquid that trickles into the extraction

chamber containing the sample. The extraction chamber was designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. At the end of the extraction process, the flask was decanted. Then, the sample was dried in an oven at 30°C.

Percentage yield

The percentage yield of the dyes was calculated using the equation below

$$\% \text{ Yield} = \frac{W2}{W1} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

Where, W1 = weight of sample

W2 = weight of dye obtained.

Melting point determination

Little quantity of the dye was poured into the melting point capillary tube and then inserted into the melting point apparatus. Temperature was raised gradually and observed carefully until it started melting. The temperature at which the melting began was recorded. The procedure was repeated twice and the average of the readings was calculated.

Preparation of sample for analysis

About 1g of dye was dissolved in a sample bottles containing 5 ml of ethanol and was taken for UV analysis and 0.05g was taken for FTIR analysis.

Spectral analysis of the extracted dye

UV-visible absorption spectral of the extracted dye

The visible absorption spectroscopic properties of the dye were recorded using caryuv-visible series spectrophotometer at the Chemistry Department, A. B. U. Zaria.

The machine was switched on and was set at a range of 400-800nm. Two cuvettes containing 2 ml of blank solution was thoroughly cleaned with a laboratory tissue and placed in the cuvette holder, one at the front holder and the other at the rear holder. The auto-zeroing commenced by pressing auto-zero key. On completion, 2ml of the dye sample was poured in a clean cuvette of the same size with the blank solution cuvette. The blank solution on the front holder was removed and replaced with the cuvette containing the sample and the door closed. The analysis commenced by pressing the start key. The result was printed out by pressing the copy key. The

spectrum of the dye sample was obtained using cary series uv-visible spectrophotometer and its molar extinction coefficient was calculated.

Determination of molar extinction coefficient of the extracted dye

The molar extinction coefficient of the extracted dye was calculated using beer-lambert equation;

$$\lambda_{max}\varepsilon = \frac{A}{Cl} \qquad \text{.......} \quad 2$$

Where **ε**= molar extinction coefficient; A= absorption at λmax; C= dye concentration in mol/litre; L= path length of the cell (1cm)

Infra-red spectral characteristics of the extracted dye

Infra-red spectral of the dye was measured on an agilent technology ftir-630s Fourier transform infra-red spectrophotometer at the Chemistry Department A. B. U. Zaria.

The machine was switched on and set for use. About 2 ml of the sample was placed on the sample holder in the machine. The start key was pressed and printed out result spectra of the dyes.

Preparation of extract concentration to be used on clinical isolate of *Klebsiella pneumonia* and *Staphylococcus aureus*

About one gram of the dye extract was weighed and added to 10 ml of 10% dimethyl sulfoxide (DMSO) to obtain 100 mg/ml stock solution of the extract. Two-fold serial dilution concentrations of 50mg/ml 25mg/ml and 12.5mg/m were prepared from stock solution. The different concentrations were labeled and kept in bijou bottles for subsequent use.

Preparation of turbidity standard and standardization of bacterial inoculum

McFarland standard was prepared by diluting 1ml of concentrated sulphuric acid with 99 ml of sterile distilled water (1%V/V). One percent (w/v) solution of barium chloride was also prepared by dissolving 0.5g of dehydrated barium chloride in 50ml distilled water. About 0.5 ml of the barium chloride solution was mixed with 99.5ml of H_2SO_4 solution to yield 1% w/v barium sulphate suspension. The turbid solution (McFarland Scale No. 1) was used as a reference to adjust the turbidity of the bacterial suspension. A colony of bacteria from an overnight growth

culture of test bacteria was added to 2ml of sterile physiological saline as suspension medium using a sterile wire loop. The bacterial suspension was compared to 0.5 McFarland standard (1.5 x 10^8 CFU/ ml) under a white background with contrasting black lines [11].

Antibacterial activity of extract on clinical isolates

Agar well diffusion method, described in CLSI manual [12] was used to determine the antibacterial activity of the extract against the clinical isolates. Micropipette was used to take about 100µl (0.1ml) of standardized inoculum of a bacterial suspension and was inoculated into Mueller Hinton agar plates (in duplicate) and spread evenly over the entire surface of the plates using a sterile cotton swab stick. The plates were left for 10 min before wells of 5mm width were dug in the agar plates using a sterile cork borer after which 100µl of the various concentrations of extract (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) were each filled in to the wells. Additional wells were filled with distilled water to serve as negative controls and ciprofloxacin (10µg) as positive control. The plates were left for 30 min at room temperature for diffusion of extracts into the agar to take place and then incubated at 37^0C for 24 h. For each bacterium tested, zones of inhibition of growth were observed and the diameter of each zone was recorded in millimeters with a transparent ruler. The means were calculated to the nearest whole number.

Determination of minimum inhibitory concentration (MIC)

The MICs of extract against the clinical isolates were evaluated using agar dilution method. The following concentrations of extracts: 12.5mg/ml and 6.25mg/ml and 3.125mg/ml were prepared by two - fold serial dilutions. 1 ml of extract concentration was added to 9 ml of Mueller Hinton agar. About 100µl each of a standardized inoculum of a test bacterium was streaked on the surface of the Mueller Hinton agar. The plates were incubated at 37^oC for 24 h. The lowest concentration of the extract which inhibited the growth of a test organism was recorded as the minimum inhibitory concentration (MIC).

Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined from the MIC plates. The plates that showed antibacterial activities at lowest concentration were streaked on freshly prepared nutrient agar plates and incubated at 37^oC for 48 h. The lowest concentration of extract that yielded no growth was the Minimum Bacteriacidal Concentration (MBC).

RESULTS AND DISCUSSIONS

Table 1: physical properties of extracted dye

YEILD (%)	COLOUR	MELTING POINT
10.9	**Red**	**145°C**

The percentage yield of the extracted dyes is 10.9%. This was expected as a major disadvantage in extraction of natural dyes is very low yield.

The colour of the dye was observed to be red and melting point was determined as145° C.

Spectral analysis

Visible absorption spectra

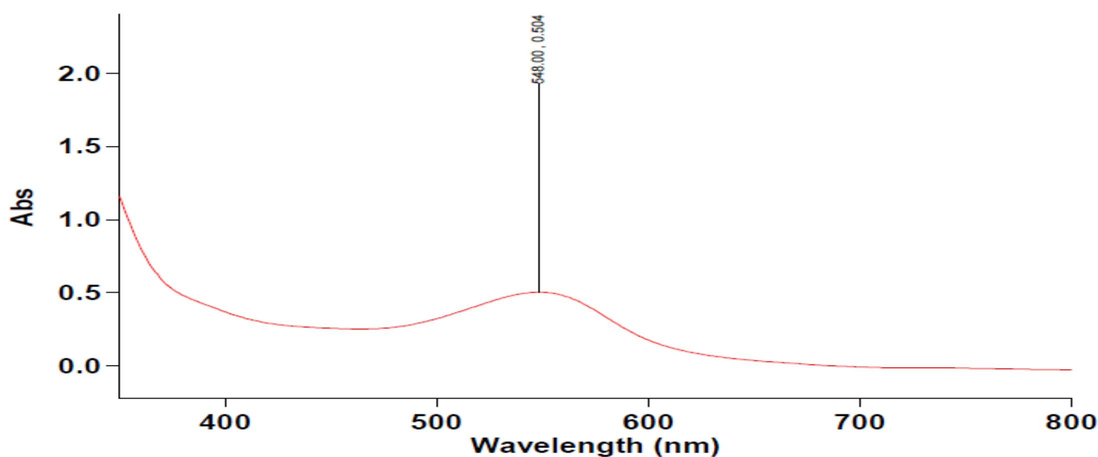


Figure 1: UV – visible spectra of the dye in

Ethanol was used to dissolve the dye sample for the UV – visible spectra analysis which gave maximum ab ction coefficient of 5.0400×10^{-4} ($mol^{-1}cm^{-1}$) using equation 2. Molar extinction coefficient of a material indicates how strongly a substance absorbs light at a given wavelength, per molar concentration.

Fourier transforms infra-red spectra of extracted dye (FTIR)

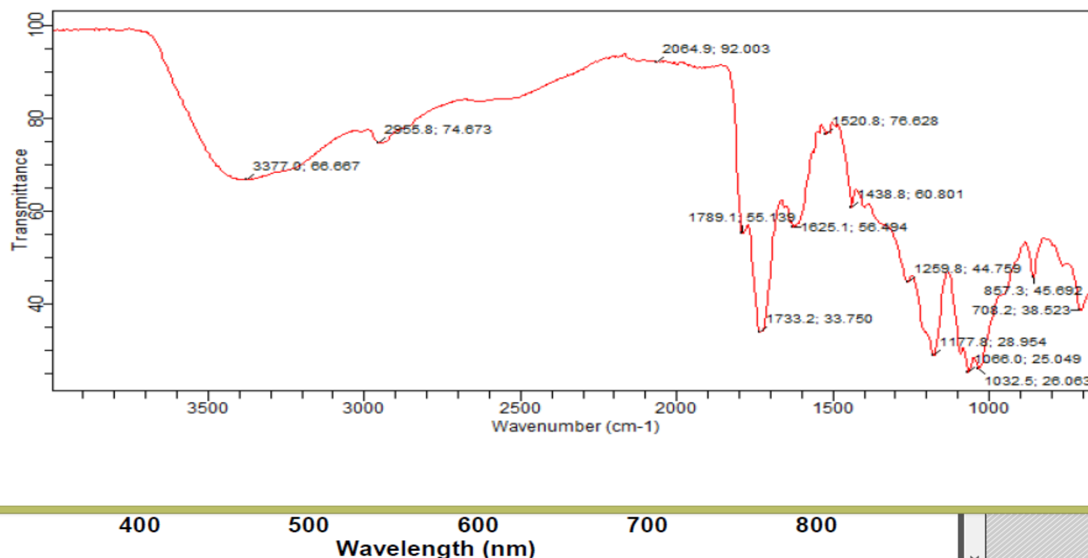


Figure 2 shows the FTIR spectrum of the extracted

The Aromatic C = C stretching is indicated at 1521 cm^{-1}, OH band is observed at 3377cm^{-1}, C = O appeared at 1733cm^{-1}, the vibration due to C-O occurs at 1260 – 1066 cm^{-1}, and the C-H vibration may be observed at 2956 cm^{-1}. Other bands correspond to bending stretches. This result is indeed in conformity with a similar work [13].

Antimicrobial activity of the dye against clinical isolates (sensitivity test).

Table 2: Sensitivity test

Bacterial isolate	Mean of zone of inhibition (mm)	Concentration in mg/ml	Positive control 10µgciprofloxacin
Klebsieller pneumonia 1	15	100	0
Klebsieller pneumonia 2	11	50	8
Klebsieller pneumonia 3	10	25	12

Klebsieller pneumonia 4	7	12.5	6
Staphylococcus aureus 1	12	100	4
Staphylococcus aureus 2	9	50	10
Staphylococcus aureus 3	8	25	16
Staphylococcus aureus 4	6	12.5	0

Table 2 shows the antibacterial activities of the extracted against *Klebsieller pneumonia* and *Staphylococcus aureus*. It was observed that there were inhibitory activities at all the concentration of the extract on the isolates. As expected the mean zone of inhibition of the isolates decreases with decrease in concentration of the extract. Musa et al.[13] observed that the higher the concentration of the dye the less resistant the bacteria are.

10µg ciprofloxacin was used as a positive control. It is interesting to note that the dye extract is more effective than the control on both the isolates, particularly at higher extract concentrations. However, the result also suggests that the extract and the control complement each other's activity on the isolates. Thus, at 100 mg/ml extract concentration the total mean zone of inhibition for the two anti – microbes on the isolates were 15mm and 16 mm for the *Klebsieller pneumonia* and *Staphylococcus aureus* respectively. The optimum result was obtained at 25mg/ml of the dye extract when the total mean zone of inhibition of the isolates reached its peak of 22mm and 24mm for the *Klebsieller pneumonia* and *Staphylococcus aureus* respectively.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the dye against the test organisms

Bacteria isolate	**MIC (mg/ml)**	**MBC (mg/ml)**
Staphylococcus aureus	12.5	25
Klebsiella pneumonia	6.25	12.5

Table 3 shows the minimum inhibitory concentration of the dye extract against the test organisms. It was found to be 6.25 mg/ml and 12.5 mg/ml while the minimum bactericidal concentration was obtained as 12.5 mg/ml and 25 mg/ml for the *Klebsiella pneumonia* and *Staphylococcus aureus* respectively.

The minimum inhibitory concentration (MIC) is the minimum concentration of the dye extract (anti – microbe) that can inhibit the growth of the bacterial (isolate) while the minimum bactericidal concentration is the minimum concentration of the dye extract that can completely kill the bacteria (microbe). This result therefore suggests that the dye extracted from hibiscus sabdariffa flower can be used as bacteriocides. Also, the MIC showed that the dye extract from hibiscus sabdariffa flower is a very potent anti-microbial extract against the test organisms at lower concentration. Therefore it may be used as preservatives on food substances against micro – organisms.

CONCLUSION

Zobo dye was successfully extracted from hibiscus flower using soxhlet apparatus with methanol as the extracting solvent. The uv-visible analysis of dye show maximum absorption at 548nm in ethanol, and from the FTIR spectrum of the extracted dye, the Aromatic C = C stretching is indicated at 1521 cm^{-1}, OH band is observed at 3377cm^{-1}, C = O appeared at 1733cm^{-1}. The vibration due to C-O occurs at 1260 – 1066 cm^{-1}, and the C-H vibration may be observed at 2956 cm^{-1}. The dye extract was tested for microbial activity against *Klebsiella pneumonia* and *Staphylococcus aureus* isolates. There were inhibitory activities at all the concentration of the extract on the isolates. As expected the mean zone of inhibition of the isolates decreases with decrease in concentration of the extract. The result also suggests that the extract and the control (Ciprofloxacin) complement each other's activity on the isolates. The best result was obtained at 25 mg/ml of the dye extract when the total mean zone of inhibition of the isolates reached its peak of 22 mm and 24 mm for the *Klebsieller pneumonia* and *Staphylococcus aureus* respectively. The minimum inhibitory concentration of the dye extract against the test organisms were found to be 6.25 mg/ml and 12.5 mg/ml while the minimum bactericidal concentration was obtained as 12.5 mg/ml and 25 mg/ml for the *Klebsiella pneumonia* and *Staphylococcus aureus* respectively.

The result therefore suggests that the dye extracted from hibiscus sabdariffa flower is a very potent anti-microbial extract against the test organisms at lower concentration and may also be used as bacteriocides.

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