Phytochemical Analysis of Anthocyanin Compounds from *Hibiscus Sabdarriffa L* and Studying their Antioxidant and Biological Activity

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Abstract
This study included the separation and diagnosis of anthocyanin compounds and testing the antioxidant activity and biological activity on two types of bacteria that infect plants (*Pseudomonas savastanoi*, *Acrobacterium tumfaciens*) from the methanolic extract that was prepared using the continuous extraction device (Soxhlet) The anthocyanin compounds were diagnosed using a high-performance liquid chromatography device HPLC. The results showed that the flowers of the *Hibiscus Sabdarriffa L* contain four anthocyanin compounds (peonidin, malvidin, delphinidin, cyanidin). The biological activity showed a high inhibitory effect against *Acrobacterium tumfaciens* bacteria at a concentration of 100 mg/cm^3 20 mm, while the highest inhibitory amount was against *Pseudomonas savastanoi* bacteria at a concentration of 400 mg/cm^3 with 15 mm compared to some antibiotics. The antioxidant activity showed the methanolic extract had an anti-oxidant effect of 186.787 µg/ml at a concentration of 500 µg/ml and was less effective by 7.232 µg/ml at a concentration of 125 µg/ml compared to ascorbic acid.

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1. Introduction

Hibiscus sabdarriffa L. is a shrubby herbaceous plant and sometimes a perennial belonging to the Malvaceae family, grown in many different parts of the world, China, Thailand, Indonesia, Saudi Arabia, Vietnam, Egypt, Sudan and Nigeria. It has been used in traditional medicine as a diuretic, analgesic, antitussive and antihypertensive. The plant extract is used to treat many neurological diseases, cardiovascular diseases, atherosclerosis, obesity, liver disorders and control of hypertension [1]. The *Hibiscus* flowers contain many active compounds such as phenols, alkaloids, terpenes and anthocyanins. Many studies have shown that anthocyanins have therapeutic properties including antioxidants, anticancer, antimicrobial, anti-inflammatory, liver and heart protective properties [2]. Anthocyanins are natural secondary metabolites that impart colour to various fruits, vegetables, and plants. These pigments are categorised as flavonoids and serve as natural antioxidants. Antioxidants are compounds that inhibit oxidation reactions caused by free radicals [3].

2. Materials and methods

Collecting Plants

After planting the *Hibiscus sabdarriffa L.* plant, the flowers were collected from the plant, cleaned, washed well and dried using a fan drying oven.

Preparation Extract of Plant

The extraction process was carried out using a continuous extraction device (Soxhlet), where the sample was ground after drying it for the purpose of extraction based on the polartiy, starting with petroleum ether and ending with methanol. If you took 50 grams of the powdered *Hibiscus sabdarriffa L* were taken and then placed in the device with the addition of 500 ml of solvent. The device was operated at a rate of 8 hours daily until the solvent used becames colorless. The process was repeated according to the polarity. The crude methanol extract was taken from the *Hibiscus sabdarriffa L* plant and the solvent was evaporated using a rotary evaporator. After that, the extracts were placed in dark, sterile glass bottles in a cool place to protect them from damage [4, 18].

Diagnosis of Separated Anthocyanin Active Compounds using an HPLC Device

Anthocyanins were diagnosed using HPLC SYKAM (Germany) where the mobile phase was an equal flow of 95/5 (v/v) mixture of water (pH 7.0) (2%), formic acid at a flow rate of 0.8 ml/min, the column type was C18-ODS (25 cm * 4.6 mm) and the UV-Vis detector at = 520 nm [5]

Biological Effectiveness

The bacteria used in the study are *Pseudomonas savastion, Acrobacterium tumfaciens.*

Agricultural media used

The nutritional agar medium was utilised in accordance with the manufacturer's specifications.

Antibiotic

Table 1: The effect of standard antibiotics on the bacteria used under study

Antibiotic	*Acrobacterium tumfaciens*	*Pseudomonas savastanoi*
Amoxicllin (AMC10)	-	-
Azithromycin (AZM15)	15	12
Trim Methbrin (TMP10)	-	-
Nalidixic acid (NA30)	10	-
Ciproder (CIP10)	20	15

(-)no effect.

Biological Efficacy Testing

The study assessed the inhibitory activity against the bacteria using the diffusion method, creating holes with a diameter of 6 mm at three replicates for each bacterium. A prepared bacterial suspension was spread and compared with the standard McFarland solution, which was subsequently incubated. Following a 30-minute incubation at 37 °C to facilitate absorption, the extract was prepared to evaluate its inhibitory activity against the studied bacteria by achieving concentrations of 400, 200, and 100 mg/cm³, derived from dissolving 1 gramme of the extract in 2.5 cm³ of dimethyl sulfoxide (DMSO) to yield a 400 mg/cm³ concentration. After that, the rest of the concentrations were made by dilution method, then the mixture was sterilized by pasteurization at a temperature of 62 for 10-15 minutes, then an amount of 0.5 microliters of each concentration was taken and injected using a micropipet into the agar holes at a rate of three replicates for each isolate and incubated at a temperature of 37 degrees Celsius in the incubator. For 16 hours [6, 19].

Antioxidant activity

DPPH test (1,1-Diphenyl-2-picrylhydrazyl) was used to determine the antioxidant activity of methanolic extract of the *Hibiscus sabdarriffa L.* [7]

DPPH (is a purple-colored organic solvent that turns yellow when in contact with a reducing agent) and the method of work was as follows:

1. A series of dilutions of the extract (15.6, 31.2, 62.5, 125, 250, 500) micrograms/ml were prepared and 10 ml of Methanol:DMSO solution at a ratio of 9:1 were added to each of them.
2. Dissolve 0.02 g of DPPH in 100 ml of Methanol:DMSO at a ratio of 9:1.
3. Add 0.3 ml of DHHP to each tube.
4. Blank negative control tubes containing DPPH solution and Methanol:DMSO only.
5. Sample tubes containing DPPH solution and Methanol:DMSO solution were prepared.
6. Then the concentrations prepared from the plant extracts were added at a rate of 3 replicates for each concentration.
7. The positive control tube was prepared containing DPPH solution, Methanol:DMSO solution and ascorbic solution.

The results were read on the spectrophotometer at a wavelength of 517 nm [12].

The results were read according to the following equation:

$$\text{Antioxidant activity \%} = [(\text{OD blank} - \text{OD sample})/\text{OD blank}] \times 100$$

OD blanlk: negative control tube

OD sample: positive sample tube [13]

3. Results and Discussion

Diagnosis of Anthocyanins compounds by HPLC

Four anthocyanin compounds were identified from the methanolic extract of the *Hibiscus sabdarriffa L.* plant. The results indicated that the malvidin compound was detected at a retention time of 3.77 minutes, while the cyanidin compound was observed at a retention time of 4.08 minutes, the delphinidin compound appeared at a retentiontime of 5.88/min, and the peonidin anthocyanin appeared at a retention time of 7.90/min as shown in Table (2) and Figures (6-1). The findings of this investigation aligned with those of study [8], which demonstrated the existence of anthocyanin chemicals in the methanolic extract of *Hibiscus* flowers, as two anthocyanin compounds were identified, cyanidin and delphinidin, which were identified by HPLC, CPC. The results of our study also converged with the study [9] that

researchers indicated that the *Hibiscus* flowers contain anthocyanin compounds through high-performance liquid chromatography analysis, and this extract showed moderate antioxidant and free radical scavenging activity and strong inhibitory effects against bacteria [20].

Table 2: Anthocyanin compounds isolated from methanolic extract of *Hibiscus sabdarriffa L*

No	Name	Ret.time	Con.%
1	Peonidin	7.90	31
2	Delphinidin	5.88	31
3	Cyanidin	4.08	16
4	Malvidin	3.77	11

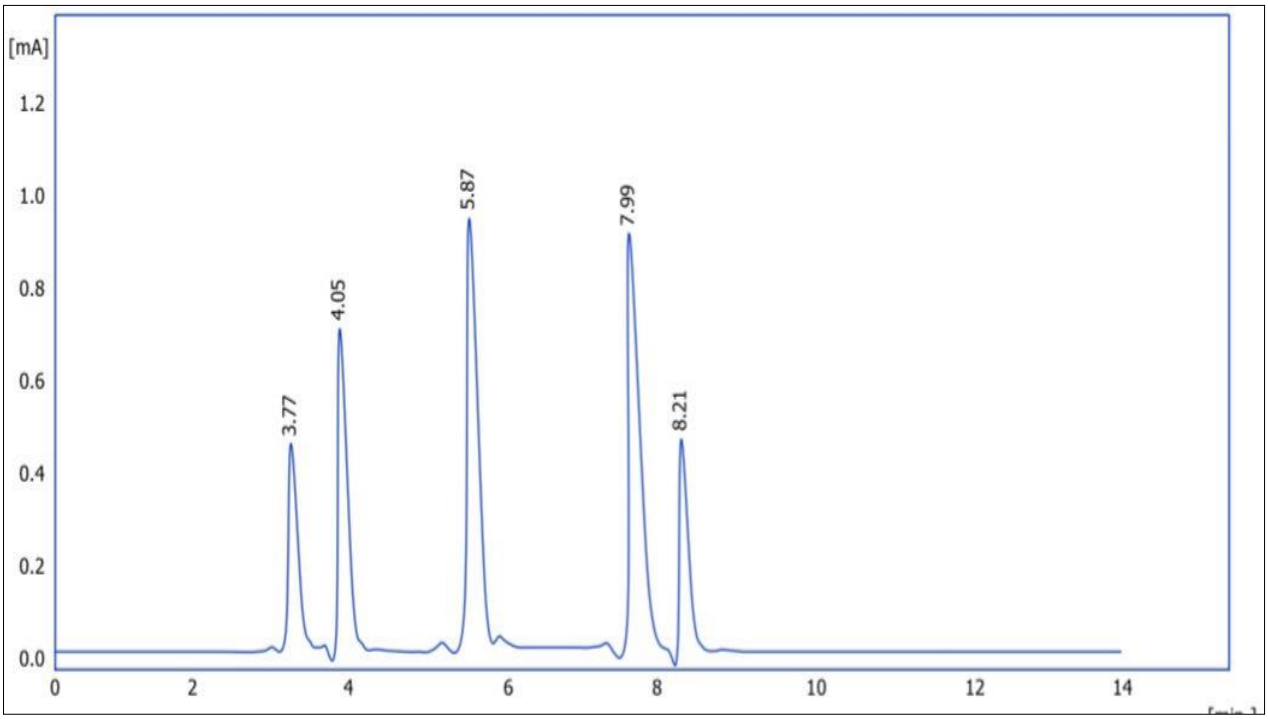

Fig 1: Curve of Anthocyanin Separated from *Hibiscus sabdarriffa L* using HPLC technique

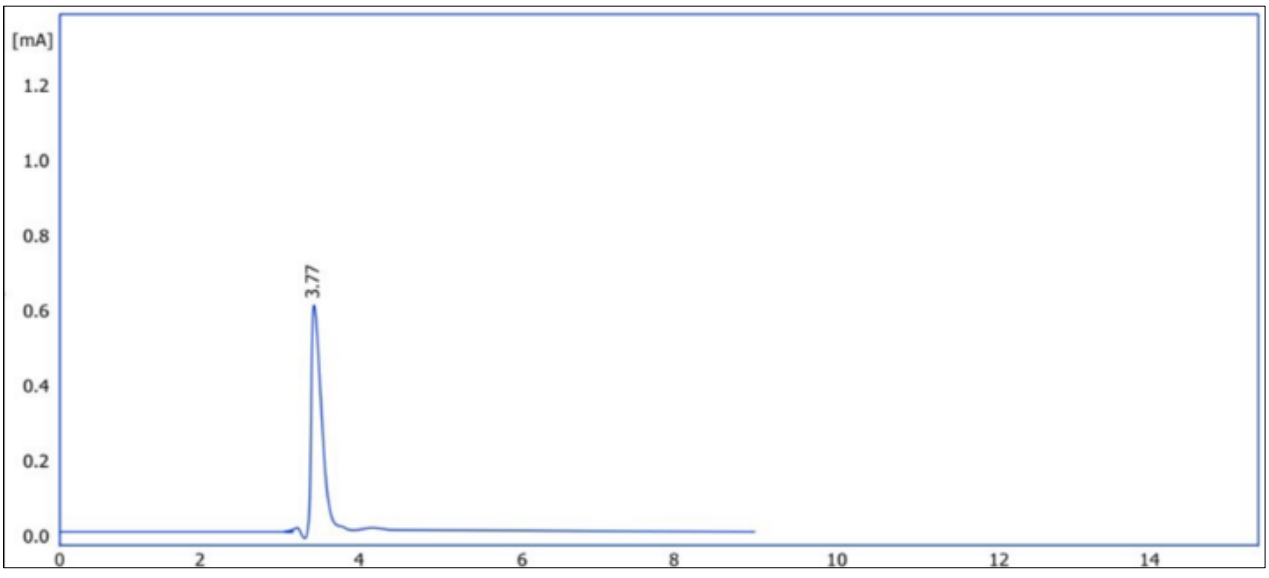

Fig 2: Standard calibration curve for malvidin using HPLC technique.

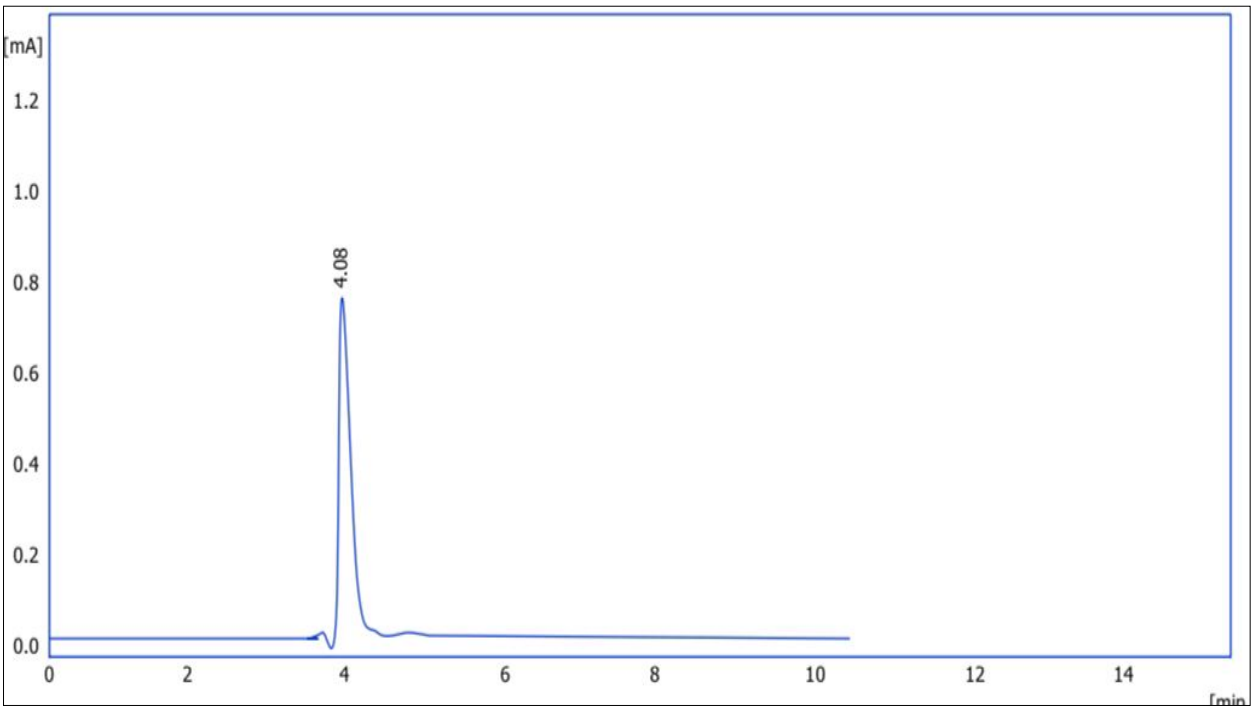

Fig 3: Standard calibration curve of cyanidin using HPLC technique

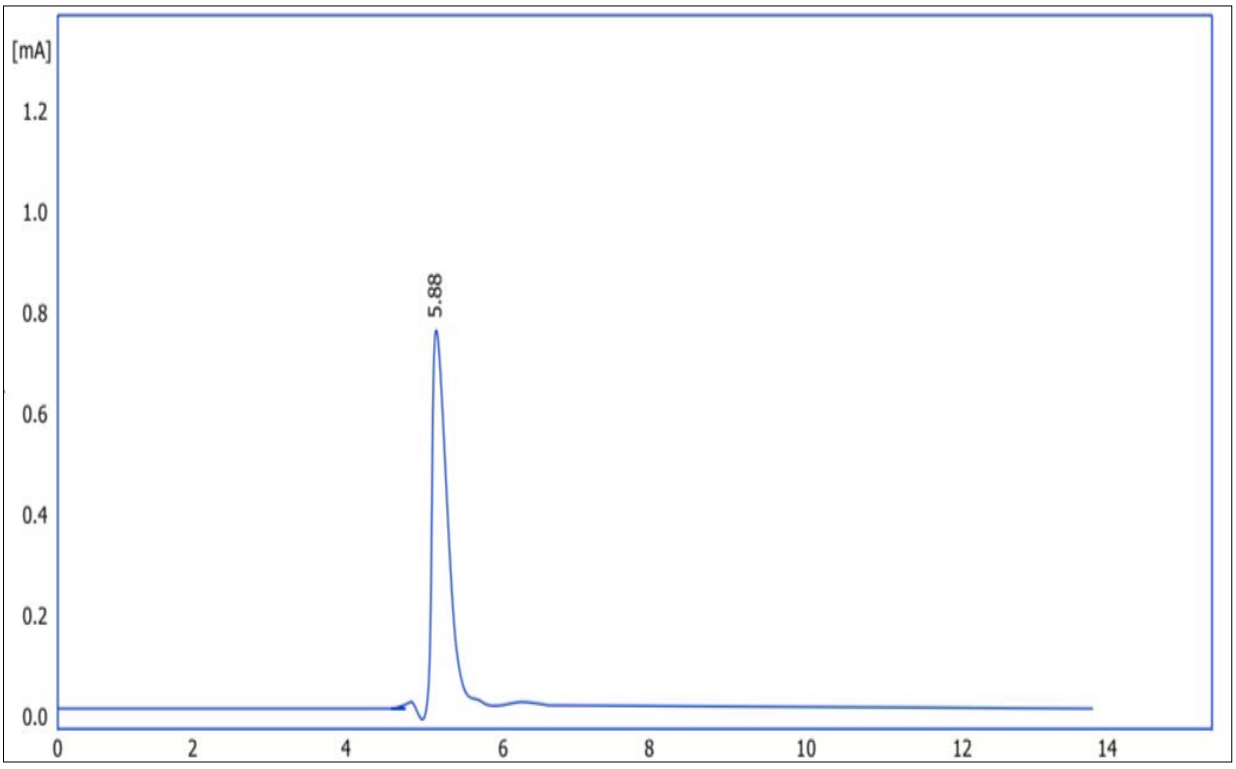

Fig 4: Standard calibration curve of delphinidin using HPLC technique.

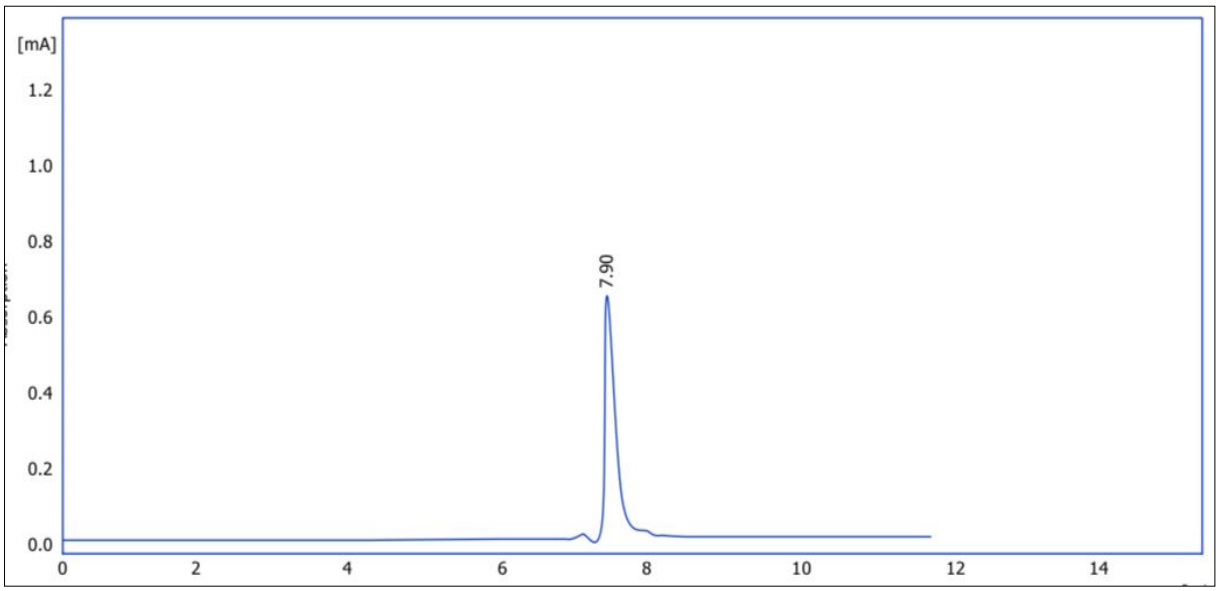

Fig 5: Standard calibration curve of peonidin using HPLC technique.

The Inhibitory Effect of Anthocyanin Isolated from the Flower *Hibiscus sabdarriffa L*

The methanolic extract of the flowers of the *Hibiscus sabdarriffa L*. showed the highest effect at a concentration of 100 mg/cm3 with an inhibition value of 20 mm against *Acrobacterium tumfaciens* bacteria, followed by concentrations of 200 and 400 mg/cm3, respectively, with an inhibition value of 16,17 mm. The concentration of 400 mg/cm^3 gave the highest inhibition value against *Pseudomonas savastanoi* bacteria with an inhibition value of 15 mm, followed by concentrations of 100 and 200 mg/cm^3 with an inhibition value of 10,9 mm, respectively, as shown in Table (3) and Figure 6 (a-b). These results were similar to the study [9], where researchers identified anthocyanin compounds in the *Hibiscus sabdarriffa L.* plant, and they had strong inhibitory effects against the bacteria *Escherichia coli*, *Staphylococcus aureus*, Staphylococcus intermedius, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, with inhibitory concentration (IC50) values in the range of 0.15 to 4.64 mg/ml and minimum inhibitory concentration (MIC) values ranging from 1.66 to 7.11 mg/ml. The study [14] showed that *Hibiscus sabdarriffa L.* extracts rich in phenolic acids, flavonoids, and anthocyanins demonstrated antibacterial efficacy against clinical isolates and foodborne pathogens, including multidrug-resistant bacteria. Anthocyanin compounds from various plants were proven to act as antibacterials by disrupting the bacterial cell wall and have activities against *Escherichia coli* and *Salmonella*. The current study was similar to with the study [15] Demonstrating that anthocyanins can impede both Gram-positive and Gram-negative serotypes of *Staphylococcus aureus* and *Escherichia coli*, in addition to the mould *Aspergillus flavus*. Laboratory studies have demonstrated that anthocyanins possess anti-inflammatory properties *in vitro*. The findings indicate that pure anthocyanins from *H. sabdariffa L.* may serve as a viable source of natural antioxidants, antibacterials, and anti-inflammatory agents in functional foods and pharmaceuticals [17, 21].

Table 3: Shows the inhibitory effect of anthocyanin compounds isolated from the *Hibiscus sabdarriffa L*

Transactions	Types of Bacteria		Extract effect
	Acrobacterium	***Psedomonas***	
400	17b±0.5	15c±0.7	16a
200	16c±0.5	10d±1	13c
100	20a±0.5	9e±0.5	15b
Effect of bacteria	18a	11b	

• Mean diameter of the circle calculated using three isolates for each bacterium.
• Distinct letters, both horizontally and vertically, denote significant differences at a significance level of 0.01≥P.
• The diameter of the inhibition circle is measured in millimetres.
• The figures following (±) denote experimental error.

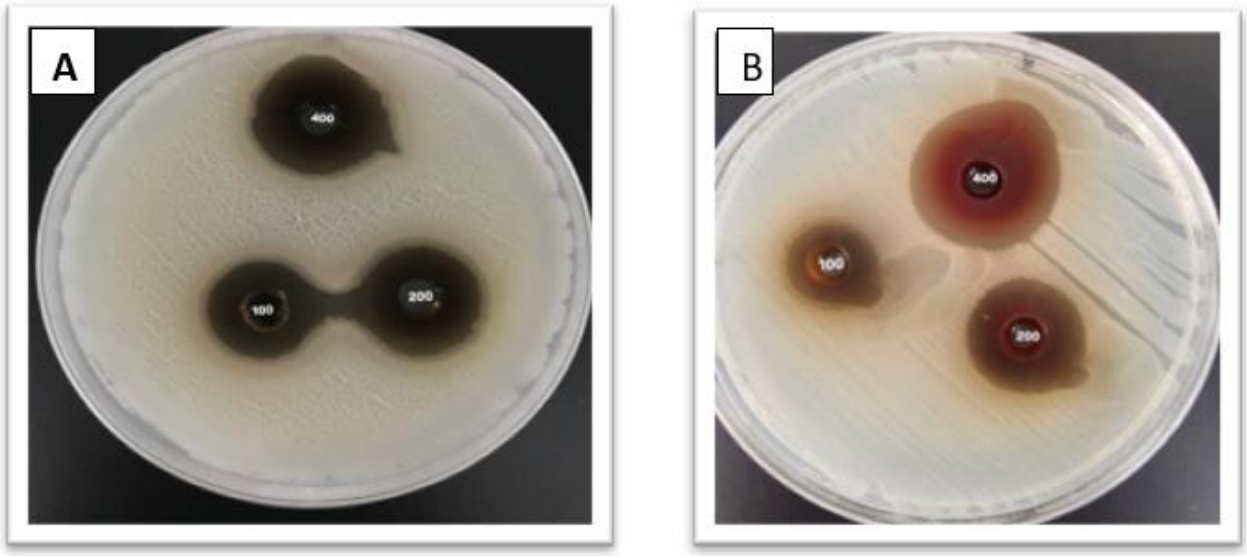

Fig 6: Shows the inhibitory effect of the methanolic extract of the flowers of *Hibiscus sabdarriffa L*

A. Effect of the methanolic extract containing anthocyanin compounds against the bacteria *Pseudomonas savastanoi*
B. Effect of the methanolic extract containing anthocyanin compounds against the bacteria *Acrobacterium tumfaciens*

Antioxidant Activity of Methanolic Extract of Anthocyanin Compounds *Hibiscus sabdarriffa L* Flowers

The results of the antioxidant activity showed that the methanolic extract content anthocyanin compounds of the *H. sabdariffa L.* plant had a high antioxidant activity at a concentration of 500 micrograms/ml, amounting to 186.787 micrograms/ml, while the least effect was at a concentration of 125 micrograms/ml, amounting to 7.232 micrograms/ml, compared to ascorbic acid, as shown in Table (4) and Figure (7). This action is attributed to the presence of anthocyanin chemicals in the methanolic extract of *H. sabdariffa L*. These findings are analogous to the research [10], which confirmed the ability of the alcoholic extract of *H. sabdariffa L.* anthocyanins to trap free radicals produced by the stable free radical, higher than the ability of the aqueous extract, as the alcoholic extract of *H. sabdariffa L.* anthocyanins recorded a total antioxidant activity of 2.8% higher than the aqueous extract of 1.9%. The study [11], which compared the effect of Antioxidant activity between two varieties of *H. sabdariffa L.* by DPPH test. Red

H. sabdariffa L. leaves showed a high percentage of free radical scavenging activity which is 85.65 ± 0.33% compared to white *H. sabdariffa L.* leaves extract 83.49 ± 0.13% at a concentration of 1 mg/ml. The researcher [16] showed that the antioxidant properties of *H. sabdariffa L.* extract rich in anthocyanins succeeded in inhibiting the production of superoxide by xanthine oxidase. This study also showed that anthocyanin extract has a strong ability to scavenge free radicals.

Table 4: Antioxidant activity of anthocyanin compoundsfrom the *Hibiscus sabdarriffa L*

التراكيز	Ascorbic acid		M.H	
15.6	13.469	0.13469	52.016	0.52016
31.2	14.421	0.14421	48.817	0.48817
62.5	26.258	0.26258	33.240	0.33240
125	26.938	0.26938	7.232	0.0723
250	54.693	0.54693	61.6134	0.61613
500	70.748	0.70748	186.787	1.8678

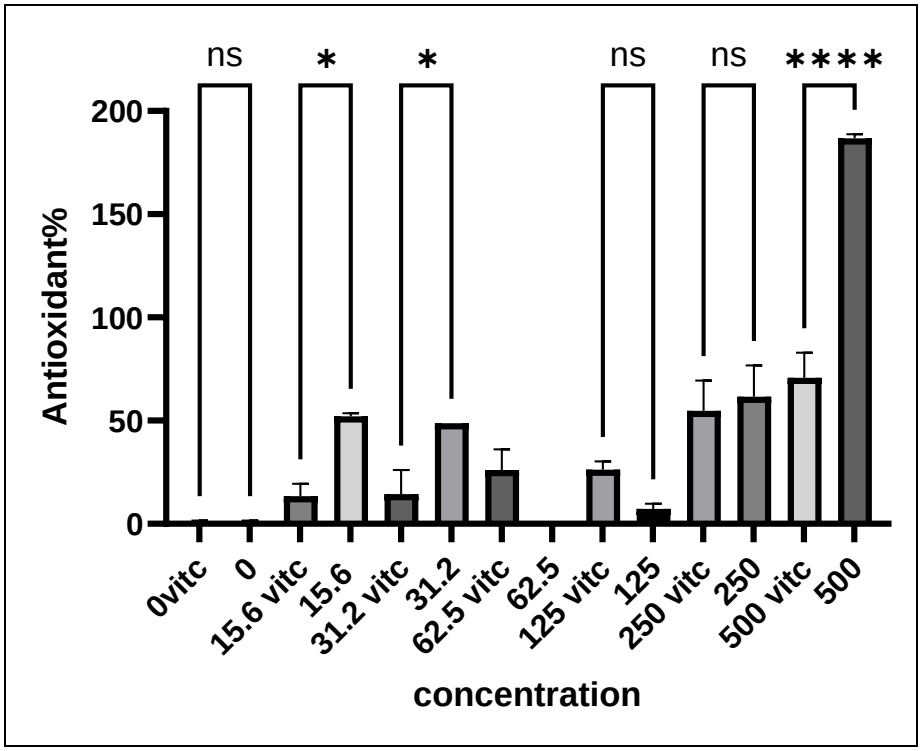

Fig 7: A diagram showing the antioxidant activity of the anthocyanin compounds from the *Hibiscus sabdarriffa L*

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