

EXTRACTION, ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS IN CALYX AND FLOWERS OF *HIBISCUS SABDARIFFA*

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ABSTRACT

The present study was based on the extraction, isolation and identification of bioactive compounds in calyx and flowers of *Hibiscus sabdariffa*. The fresh calyx and flowers of *H. sabdariffa* were procured from the Ludhiana Punjab region, India and made authenticated from the Botanist Dr S Shweta, Asst. Prof., Guru Ghasidas, Vishwavidyalaya, Bilaspur, with ref. no. Bot./GGV/2025/156. The dried calyces & flowers of *H. sabdariffa* were minced and immersed in absolute ethanol for 3 days separately and then filtered. The left fibres were repeatedly extracted by ethanol three times. Alcohol was removed from the filtrate using the rotary vacuum evaporator. After preliminary phytochemical screening, the biochemical moieties were isolated using HPLC methods and structure elucidated using IR, NMR and Mass spectrum analysis. It concluded that this study shows that ethanolic calyx and flowers extract of *H. sabdariffa* contain flavonoids (quercetin) organic acid (citric acid) and phytosterols (β -sitosterol) in abundance. Moreover, calyx is rich in diverse bioactive compounds i.e., flavonoids, saponins, steroid, tannins, phenol, terpenoids, glycoside and carbohydrate. Due to these versatile phytoconstituents *H. sabdariffa* has been reported for various pharmacological properties i.e., anti-inflammatory, anti-tumor, anti-hypertension, anti-obesity, anti-microbial, anti-oxidant, delayed puberty, neuro-protective, hepatoprotective and anti-diabetic. It suggests confirming the therapeutic uses of *H. sabdariffa* by identifying the specific flavonoids present in the calyx and flowers of the plant.

KEYWORDS: *Hibiscus sabdariffa*, isolation, bioactive compounds, HPTLC, FTIR.

INTRODUCTION

Hibiscus sabdariffa (Roselle) is cultivated in India in an area of approx. 1.5 lacs hectares with production around 11q/ha¹. *Hibiscus sabdariffa* is a shrub that belongs to the Malvaceae family. It is also grown in Sudan, America, South Africa, and others, for a variety of purposes, i.e., medicine, nutrition, fiber gathering, and food consumption².

The plant has dark green to crimson stem with 3.5 meters height. The calyx is usually red and consists of 5 large sepals with a collar (epicalyx) that is 8-12 thin and has pointed tracts around the base. The leaves are green (7.5-12.5 cm long) with reddish veins and long/short petioles. In its immature state, the fruit is a green, velvety capsule with length of 1.25-2 cm^{3,4,5}.



Dried calyx



Flower

Fig 1. Different parts of *Hibiscus sabdariffa*

Taxonomy

Kingdom-	Plantae
Order-	Malvales
Family-	Malvaceae
Genus-	<i>Hibiscus</i>
Species-	<i>sabdariffa</i>

Delphinidin 3-sambubioside and cyanidin 3-sambubioside are the two main anthocyanins found in red calyces. The calyx of *H. sabdariffa* (Roselle) also contains organic acid, minerals, amino acids, carotene, vitamin C, and total sugar. Depending on the kind and region, there are different amounts of leaves and seeds. The extraction, isolation, and identification of bioactive chemicals in the calyx and flowers of *Hibiscus sabdariffa* served as the foundation for the current investigation.

MATERIALS AND METHODS

Experimental requirements

Fresh calyx and flowers of *H. sabdariffa*, ethanol 99.9%, sterile saline, UV spectrometer, micropipette, pH meter, HPLC, and heating mantle.

Collection and authentication of plant material

The fresh calyx and flowers of *H. sabdariffa* were procured from the Haridwar Uttarakhand Region, India and made authenticated from the Botanist Dr S Shweta, Asst. Prof., Guru Ghasidas, Vishwavidyalaya, Bilaspur, with ref. no. Bot./GGV/2025/156.

Preparation of extract

The dried calyx & flowers of *H. sabdariffa* were grinded and poured in to absolute ethanol for 3 days, separately and filtered. The left fibres were repeatedly extracted using ethanol thrice. Rotary vacuum evaporator utilized to make the extract solid. By this procedure, the dried extract powder of calyx and flowers of *H. sabdariffa* was obtained and % yield was calculated. From both the extracts, the number of phytochemicals were isolated using HPLC method. The water extract made from HS-EE dried powder was further used for animal studies⁷.

Preliminary phytochemical screening^{8,9}

Detection of Alkaloids

Before being filtered, each extract is dissolved in diluted HCl.

Mayer's Test: The filtrate treated with Mayer's reagent. The production of a yellow precipitate shows that there are alkaloids present.

Wagner's Test: The filtrates treated with Wagner's reagent. When a brown or reddish precipitate appears, alkaloids are present.

Hager's Test: Filtrates were treated with Hager's Reagent. Formation of yellow ppt indicates the presence of alkaloids.

Detection of Glycosides

Fehling's test: Using water, Fehling's solutions A & B were heated for 1 min. The transparent blue solution was mixed with eight drops of plant extract. It was then combined with 1ml of Fehling's solution and cooked for 5 minutes in a water bath. Brick-red precipitation indicates the presence of glycosides.

Detection of Saponins

Herbal extract mixed with 10ml water and agitated quickly to make a stable, long-lasting foam. Foam shows that saponins are there.

Detection of Tannins

Ferric chloride test: In a test tube, 20ml of water is used to boil 0.5g of the dried powdered sample, which is subsequently filtered. After adding a few drops of 0.1% FeCl₃, the color is tested to see if it is brownish green-black or blue-black.

Lead acetate test: Mix 2g extract with 2ml distilled water. The mixture is shaken well after 0.01g of lead acetate is added. When white turbidity and precipitate form, tannins are present.

Detection of Flavonoids

NaOH test: A tiny quantity of extract is treated with aqueous NaOH and HCl, and the development of a yellow-orange hue is monitored.

H₂SO₄ test: A part of the extract is treated with conc. H₂SO₄, and the color change to orange is watched.

Detection of terpenoids

Mix 2.0 ml of chloroform with 5 ml of the plant extract in water. Then, add the mixture to the water route, let it evaporate, and boil it with 3 ml of concentrated H₂SO₄. When the terpenoids grew, a gray color appeared.

Detection of Steroids

The 5g extract is mixed with 2ml of chloroform and conc. H₂SO₄. The red color that appeared in the bottom chloroform layer shows that steroids are present

Estimation of reducing sugars and carbohydrates

Molisch's test

Put 2–3 ml of extract of different solvents in a test tube and add a few drops of α -naphthol solution in alcohol. Then, shake the test tube and add conc. H_2SO_4 from the sides. violet ring where two liquids touch.

Fehling's test

It is employed to detect falling sugar levels. In 500 milliliters of distilled water, dissolve 34.66 kg of copper sulfate (solution A). 50g of sodium hydroxide and 17.3 grams of potassium sodium tartrate are combined with 50ml of distilled water to create Solution B. Combine two solutions in equal parts before using. Fehling's A and B solutions should be boiled together for one minute. Equal parts of the test solution should be added. Cook for five to ten minutes in a kettle of boiling water. It turned brick red after being yellow at first.

Isolation of bioactive compounds¹⁰

HPTLC Analysis

➤ Selection of stationary phase

Usually, a thin coating of silica gel or another adsorbent serves as the stationary phase. The polarity or non-polarity of the sample components determines the stationary phase selection.

➤ Selection of Mobile Phase

Depending on the analytes' solubility profile, a combination of solvents may be used as the mobile phase. A combination of polar and non-polar solvents can be used as a common mobile phase to achieve the best possible component separation.

➤ Sample preparation

Sample was prepared by dissolving the analyte in a suitable solvent. Filtration might be necessary to remove any matter.

➤ Application of sample

The sample was applied to the TLC plate in small volumes using a micropipette or a syringe. Sample application was kept uniform and concentrated in one area.

➤ Chromatographic Separation

The plate was placed in a developing chamber, where the mobile phase rises due to capillary action. The components of the herbal extract started moving with different rate on the basis of interaction with the stationary phase. Thus, components got separated.

➤ Detection

Following development, the plate was dried and examined using staining methods or UV light for colorimetric examination. Densitometric scanning, which measures band intensity, was also used to quantify the separated components.

Structural elucidation of bioactive compounds

➤ Infrared Spectroscopy

Infrared spectroscopy is effective tool for determination of different functional groups and prediction of chemical structure. The primary advantage of infrared over other methods is its ease of producing fingerprints (1300-650/cm) of the structure of molecules. No two compounds share the same fingerprint region. This technique is based on the chemical's molecular vibration, which makes every bond vibrate at a certain frequency that matches the infrared wavelength¹¹.

➤ NMR Spectroscopy

The interaction b/w extract and electromagnetic forces observed by placing extract into 2 magnetic fields, one of which is fixed and the other of which fluctuates at a radio frequency. A radio frequency detector and amplifier detect absorption as a change in signal developed, and the sample detects energy at specific combinations of fields. This absorption energy can be associated with the magnetic dipolar nature of a spinning nucleus. NMR is helpful in determining the molecule's structure. A Bruker Ultraspec 500MHz spectrometer used to plot ¹H NMR spectra in CDCl₃ and d₆-DMSO¹².

➤ Mass Spectroscopy

This technique involves repeatedly striking individual molecules with a strong electron beam. The molecules break up into a variety of fragments after being ionized, some of which are positive ions. Every form of ion has a different mass-to-charge ratio, or m/e. Since most ions only have one charge, their molecular mass and m/e ratio are equivalent. When moving ions pass through a system of magnetic and electric fields and reach a detector, signals are detected and recorded to produce mass spectra¹³.

RESULTS AND DISCUSSION

Percentage yield

The percentage yield was calculated as 16.29 % and 14.67 % in the ethanolic calyx extract and ethanolic flowers extract of *H. sabdariffa*, respectively.

Organoleptic properties

Table 1 Organoleptic properties

Ethanolic extract of <i>H. sabdariffa</i>	Organoleptic properties		
	Appearance	Color	Odor
Calyx extract	Powder	Bright red	Characteristics
Flowers extract	Powder	Off-white	Characteristics

Preliminary phytochemical screening

Table 2. Phytochemicals of ethanolic calyx extract and ethanolic flowers extract of *H. sabdariffa*

Phytochemical	Ethanolic calyx extract of <i>H. sabdariffa</i> (ECHS)	Ethanolic flowers extract of <i>H. sabdariffa</i> (EFHS)
Flavonoid	++	++
Protein	++	+
Saponins	+++	++
Phytosterol	+	—
Steroid	+	—
Tannins	++	++
Phenols	++	+
Terpenoids	+++	+++
Glycoside	++	++
Carbohydrate	+	+

Absent (-), Moderate (++), Abundance (+++)

Isolation of bioactive compounds: HPTLC analysis

Table 3. HPTLC analysis of herbal extract

Herbal extract	Solvent system	R _f value	Expected compound
ECHS	n-hexane + acetone + ethyl acetate (60:20:20)	0.78	Flavonoids (Quercetin), Organic compounds (Citric acid)
EFHS	n-hexane + acetone + ethyl acetate (60:20:20)	0.73	Phytosterol (β-sitosterol)

Structural elucidation of bioactive compounds

FTIR Spectroscopy

1. Quercetin

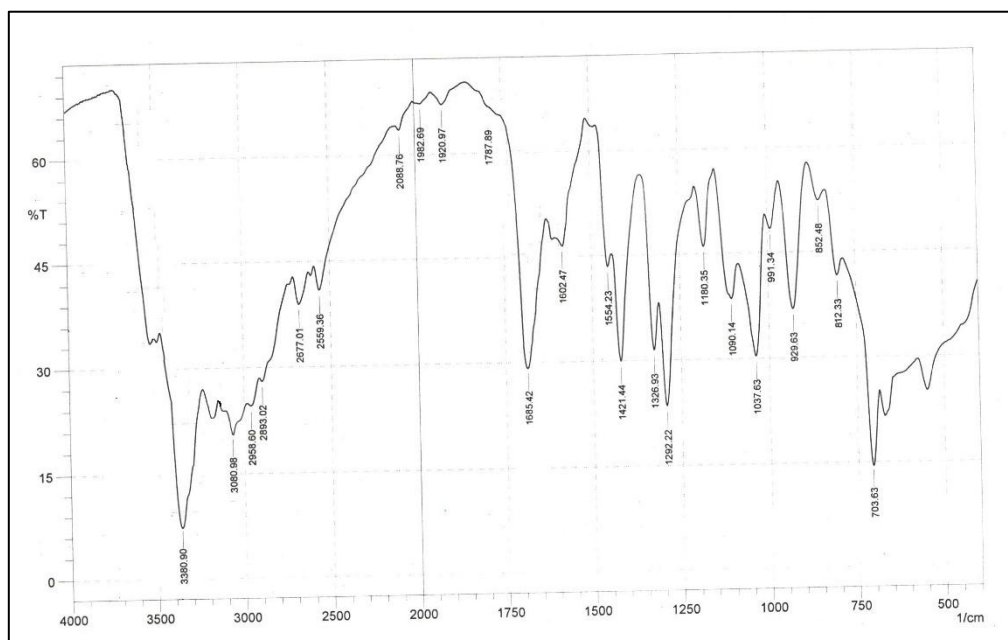
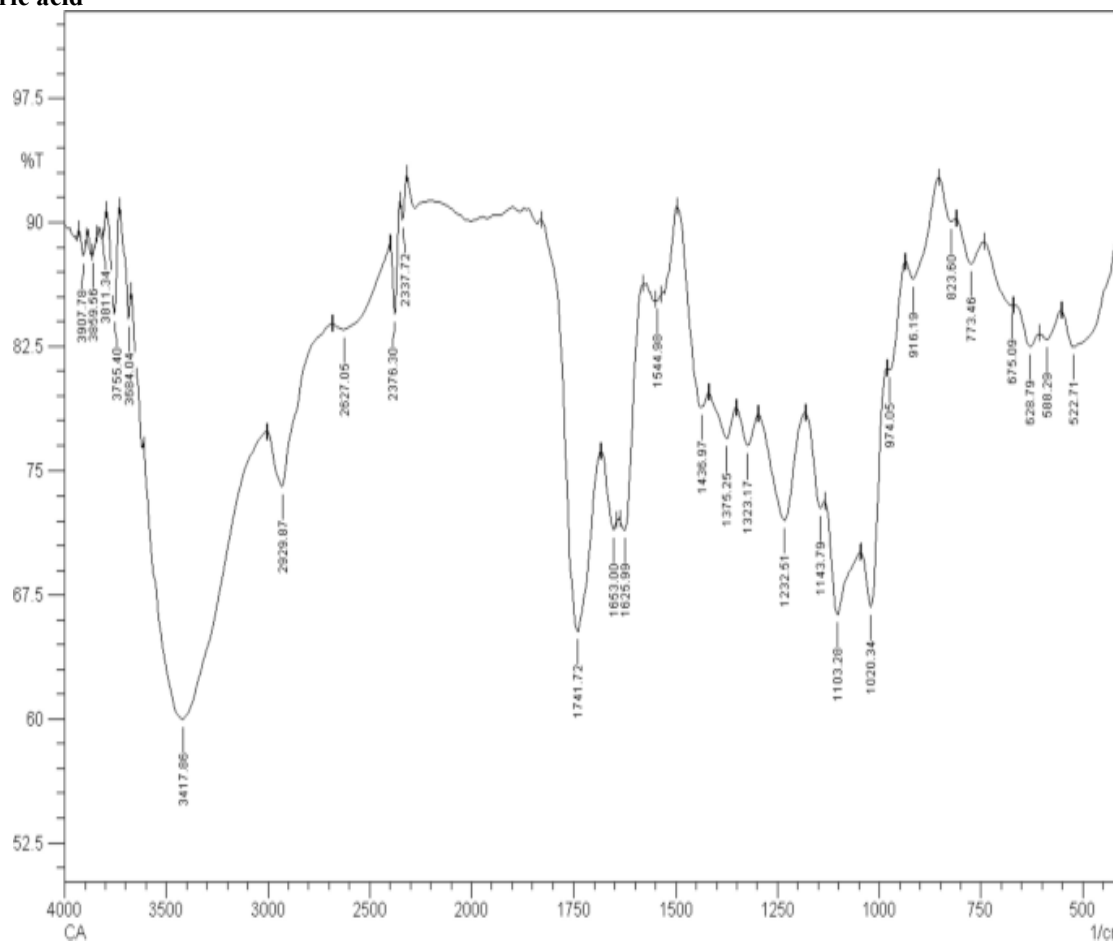


Fig. FTIR Spectrum of Quercetin

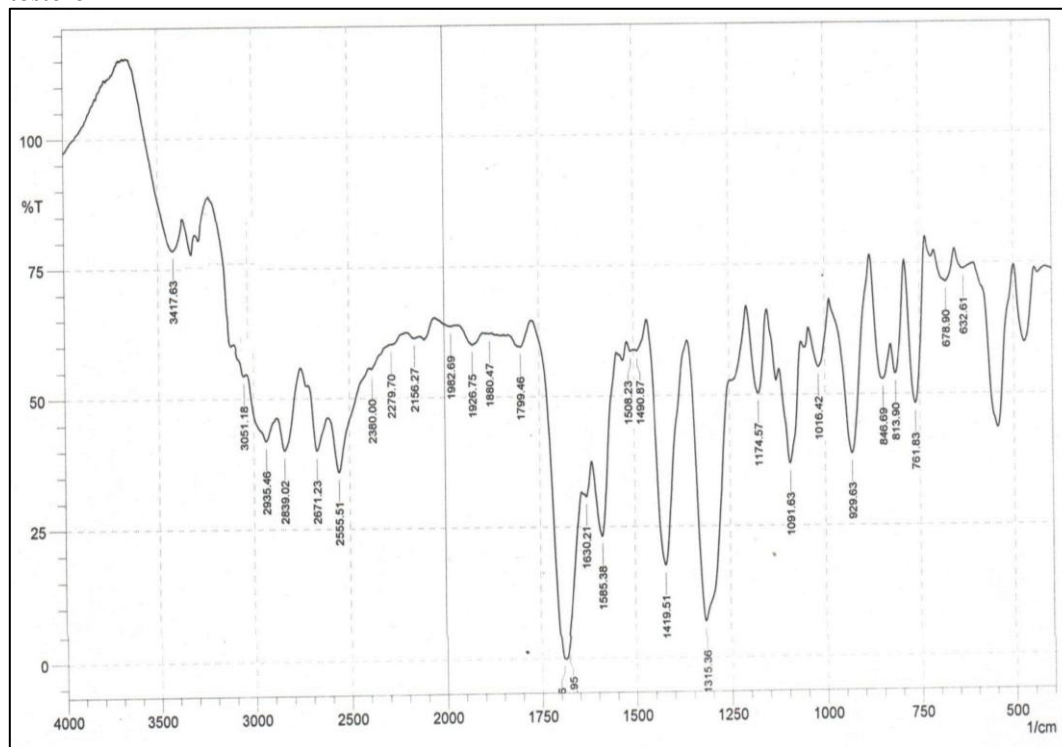
FTIR spectrum analysis:IR (KBr): 3380 (O-H str.), 3080 (Aromatic C-H str.),2958 (Aliphatic C-H str.), 1292 (C-O-H str.), 1685 (Aromatic C=C str.), 1554 (C=O str.), 1421 (C-O-C str.), 1326 (C-O str.).

2. Citric acid



FTIR spectrum analysis:IR (KBr): 3755 (O-H str.), 3417 (Ar.C-H str.),2929 (H-C-H str.), 2627 (C-O-H str.), 2376 (Aromatic H-C-H str.), 1741 (C=O str.), 1625 (C=O str.), 1103 (C-O str.), 1020 (C-N str.).

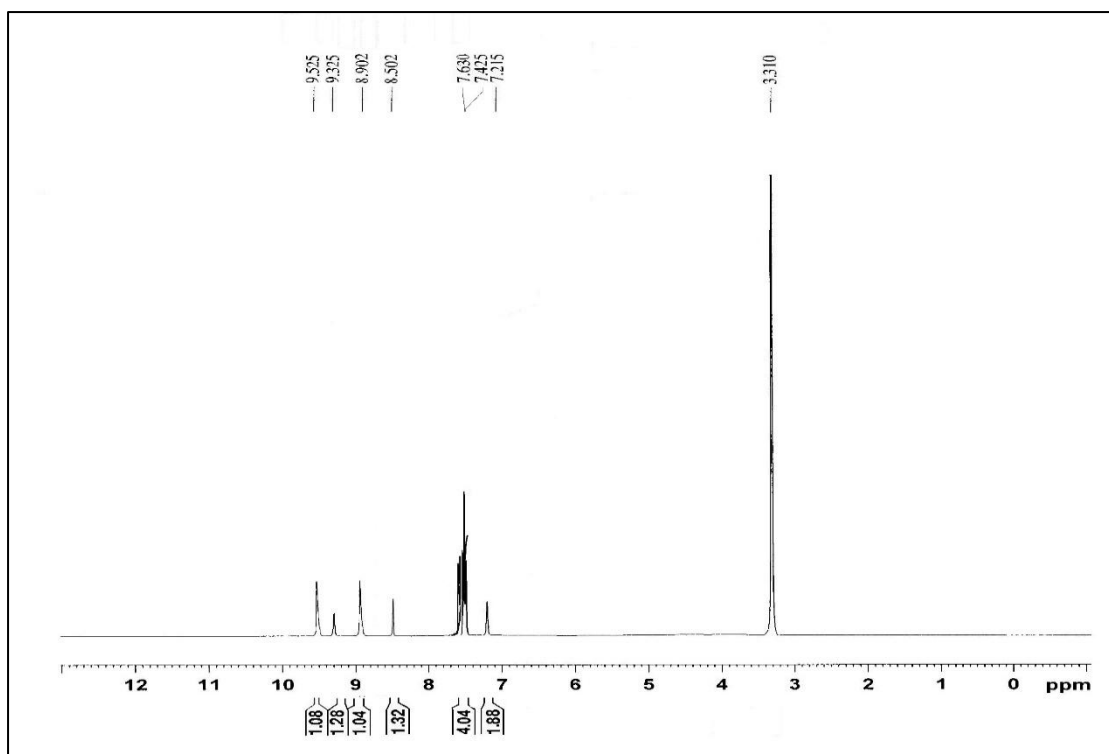
3. β -sitosterol



FTIR spectrum analysis: IR (KBr)v: 3417 (O-H str.), 2935 (C-H str. in CH₃), 2839 (C-H str. in CH₂), 1630 (C=C str.), 1419 (C-O str. in Secondary alcohol), 1315 (C-H deformation in gem dimethyl).

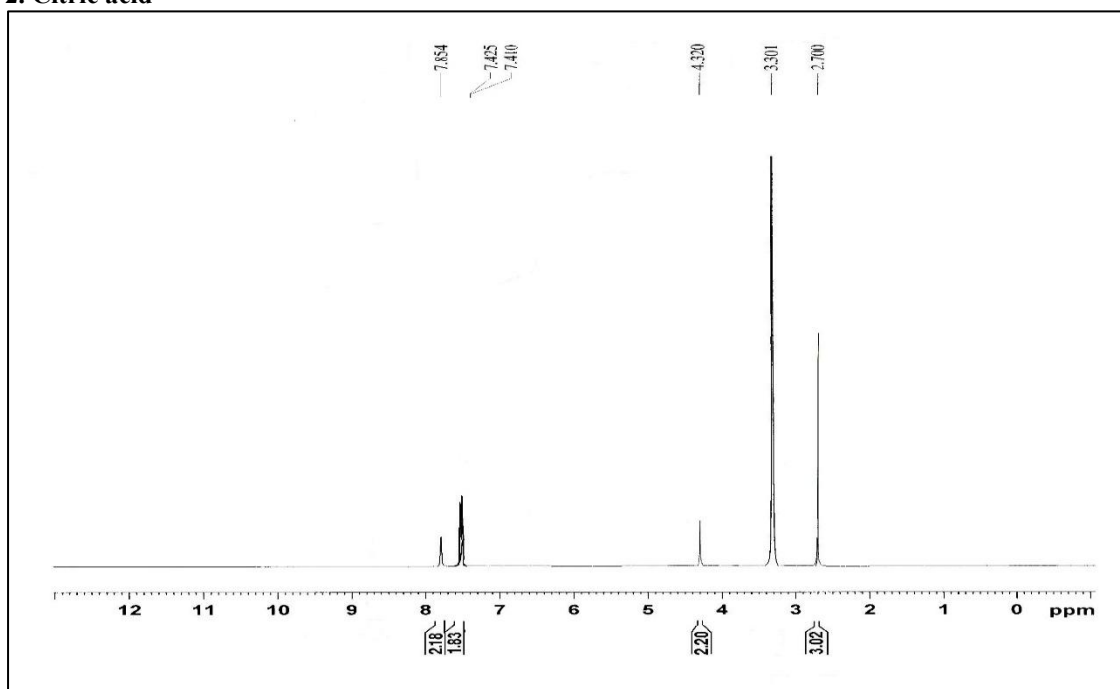
NMR Spectroscopy

1. Quercetin



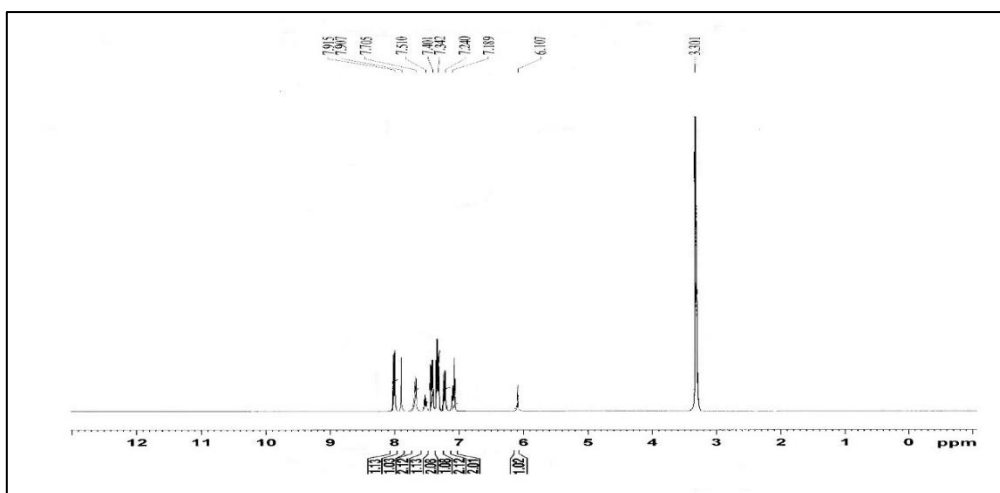
^1H NMR (DMSO-d_6): δ 7.215 (s, OH), 7.425-7.630 (m, Ar-H), 8.502 (s, OH), , 8.902 (s, OH), 9.325 (s, OH), 9.525 (s, OH).

2. Citric acid



^1H NMR (DMSO-d_6): δ 2.700 (s, CH_3), 4.320 (OH), 7.409-7.419 (Ar-H), 7.854 (OH).

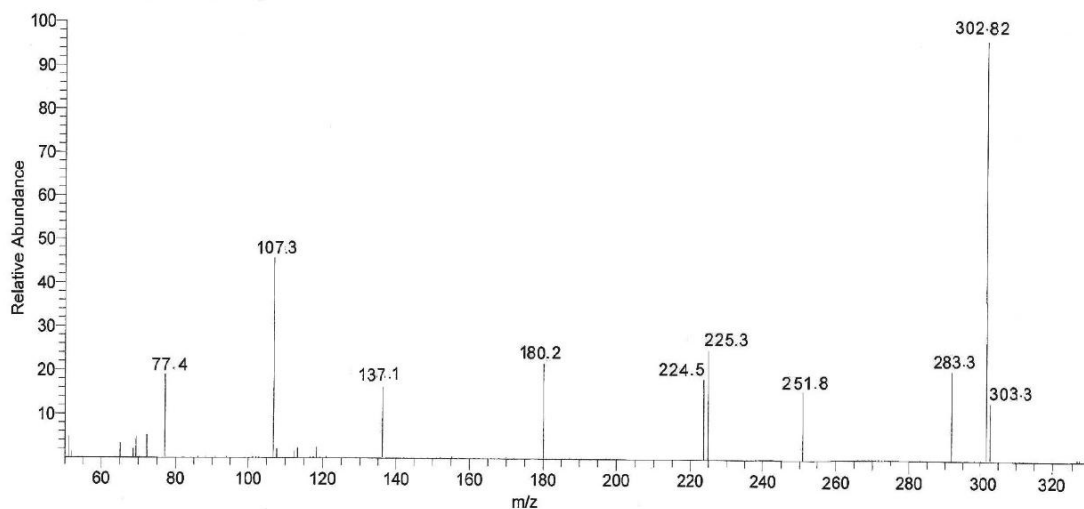
3. β -sitosterol



¹H NMR (DMSO-d₆): δ 6.107 (s, OH), 7.189 (s, CH₃), 7.240 (s, CH₃), 7.342-7.401 (m, Ar-H), 7.510 (s, CH₃), 7.705 (s, CH₃), 7.907 (s, CH₃), 7.915 (s, CH₃).

Mass Spectroscopy

1. Quercetin

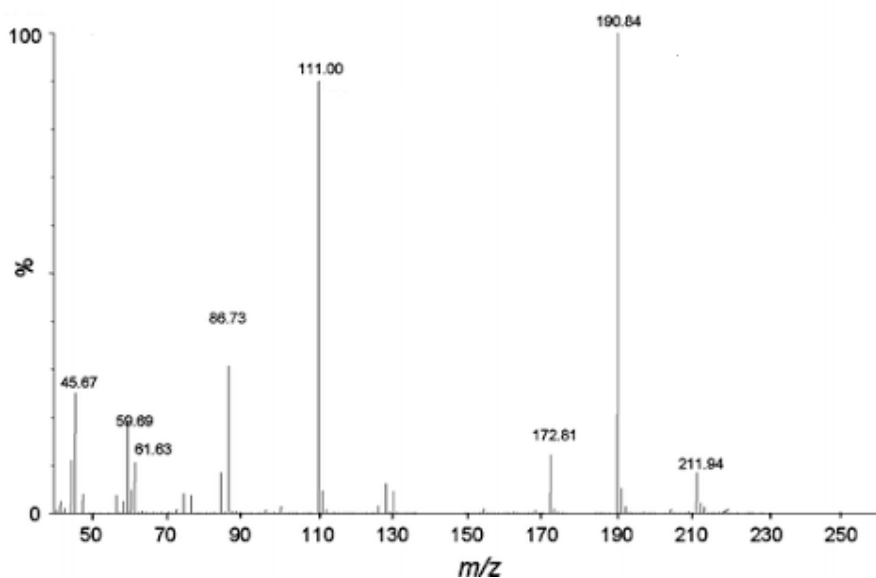


Mass spectrum of Quercetin

MS (ESI) m/z [% rel. abundance]: 302 (100) [M]⁺, 303(18) [M+1]⁺

Fragments: 283, 251, 225, 224, 180, 137, 107, 77.

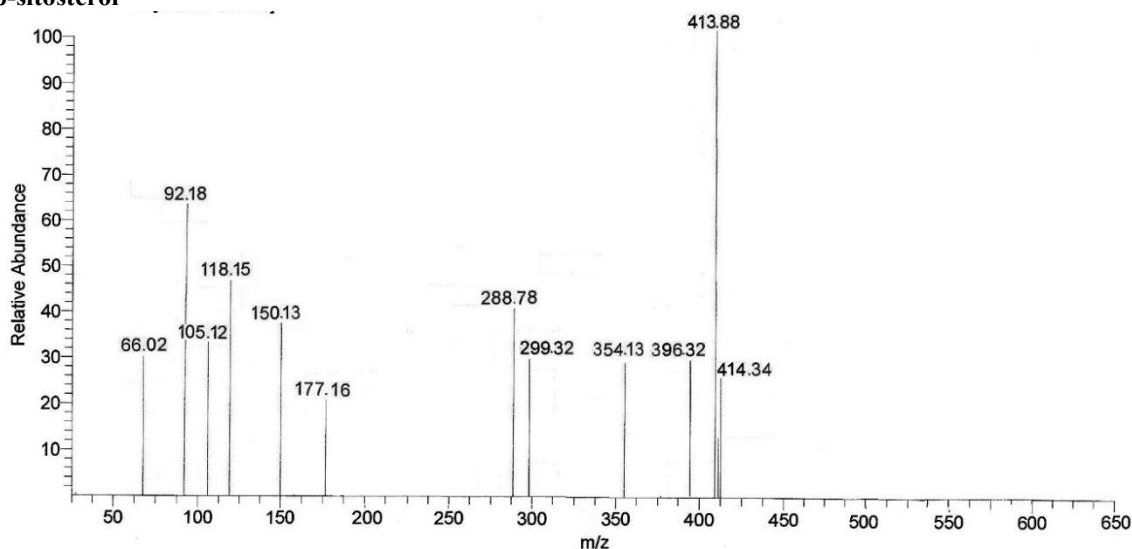
2. Citric acid



MS (ESI) m/z [% rel. abundance]: 190 (100) [M]⁺, 211 (09) [M+1]⁺

Fragments: 172.61, 111, 86.73, 61.63, 59.09, 45.67.

3. β -sitosterol



MS (ESI) m/z [% rel. abundance]: 413 (100) $[M]^+$, 414 (30) $[M+1]^+$

Fragments: 396, 354, 299, 288, 177, 150, 118, 105, 92, 66.

The results of the experiment showed that a few significant phytochemicals were present in the ethanolic extract of *Hibiscus sabdariffa* bark. The phytochemical screening revealed that the sample included alkaloids, flavonoids, and carbohydrates. Hydrocarbon compounds have been shown to possess antibacterial properties^{14,15}. To determine the total phenolic compounds, the sample's reducing ability was assessed using the Folin–Ciocalteu Reagent (FCR). The antioxidant qualities of phenolic compounds are well known, and they are essential for scavenging free radicals and preventing oxidative damage.

The phenolic content discovered in this study indicates that *Hibiscus sabdariffa* bark is a major source of phenolic compounds. Further research is required to distinguish and characterize each of these phenolic chemicals in order to fully understand their distinct contributions to the extract's overall biological activity. All things considered, the *Hibiscus sabdariffa* bark extract's phenolic content offers prospective medical applications, especially with regard to inflammation and oxidative stress¹⁶.

The plant's flavonoid components may have therapeutic uses^{17,18}. Functional group-containing flavonoids exhibit antioxidant potential and inhibit the angiotensin I-converting enzyme and lipid peroxidation^{19,20}. This study found that the ethanolic extract of *Hibiscus sabdariffa* bark contains 5.896 mg/g of flavonoids, which are comparable to quercetin. The quantity we found indicates that this plant may be a significant source of these advantageous substances. It's interesting to note that the majority of studies on *Hibiscus sabdariffa* have concentrated on its leaves, or calyces, which are likewise high in flavonoids. Our results show that although the bark has a high quantity of flavonoids, it hasn't received nearly as much attention²¹. Phytochemical experiments revealed that *Hibiscus sabdariffa* contained specific minerals and phytonutrients. The plant sample contains phytonutrients, also known as phytochemicals, such as flavonoids, glycosides, phytosterols, and tannins, while the macronutrients include proteins, lipids, oil, and diminishing amounts of sugar and carbohydrates²². The study also discovered phytosterols, which are crucial in blocking the intestinal absorption of cholesterol. According to research, β -sitosterol is one of the phytosterols present in zobo F. β -sitosterol has been used to lower serum cholesterol levels in individuals with hypercholesterolemia²³.

CONCLUSION

It concluded that this study shows that ethanolic calyx and flowers extract of *H. sabdariffa* contain flavonoids (quercetin) organic acid (citric acid) and phytosterols (β -sitosterol) in abundance. Moreover, calyx is rich in diverse bioactive compounds i.e., flavonoids, saponins, steroid, tannins, phenol, terpenoids, glycoside and carbohydrate. Due to these versatile phytoconstituents *H. sabdariffa* has been reported for various pharmacological properties i.e., anti-inflammatory, anti-tumor, anti-hypertension, anti-obesity, anti-microbial, anti-oxidant, delayed puberty, neuroprotective, hepatoprotective and anti-diabetic. It suggests to confirm the therapeutic uses of *H. sabdariffa* by identifying the specific flavonoids present in the calyx and flowers of the plant.

CONFLICT OF INTEREST

Authors declare for none conflict of interest.

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