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Investigation for determination of therapeutic potential for antitubercular activity with special reference to *Caesalpinia crista* fruits

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Abstract: In recent years, plant biochemistry has played a significant role due to plant-derived products' phytochemical details. One of the difficulties of phytochemistry nowadays is performing the aforementioned procedures with limited material due to the wide variety and low yield of phytochemicals. The secondary metabolites found in *Caesalpinia crista* have a wide range of applications. To better understand the antitubercular properties of *Caesalpinia crista*, a major medicinal plant, this study examines the most essential compounds in its fruits. Results from both quantitative and phytochemical screening revealed high concentrations of a wide variety of bioactive compounds, including alkaloids, phenols, amino acids, flavonoids, saponins, tannins, proteins, terpenoids, and glycosides. Microplate-based Alamar blue assay demonstrates their efficacy against TB. In addition, the results confirmed the presence of antitubercular potential in specific phytochemicals extracted from *Caesalpinia crista* fruits.

Introduction

Over time, nature has provided many benefits to human health, including the materials for early attempts at therapeutic intervention. People in the past often turned to plants for relief from a wide range of medical conditions. Plants have long been studied for potential of new medicines, and many different plants have been used as models for developing new pharmaceuticals, food additives, agricultural chemicals, and industrial chemicals, making plant-derived products an invaluable tool in the fight against disease (Borris, 1996). The photochemical defence mechanism relies on bioactive compounds present naturally in plants. There are two types of phytochemicals, known as main and secondary components, and each serves a specific purpose in plant metabolism. Among 2.5–5 million plant species worldwide only a small percentage of the estimated have had their phytochemicals investigated (Zambari et al., 2023).

Both plant biochemistry and organic chemistry now provide special attention to the phytochemical analysis of plant products. It is concerned with the wide range of organic compounds that plants store, and it contributes to our understanding of their chemical composition, distribution, and biological function. Understanding phytochemistry requires both the effective application of tried and true methodologies and the constant development of novel approaches to meet new difficulties. A major challenge in phytochemistry is carrying out all of these processes with only a small amount of starting material. To address a biological problem linked to plant growth control, the biochemistry of plant-animal interactions, or the evolution of ancient plants, it is usually necessary to identify a wide range of complicated chemical compounds that may only be available for analysis in microgram levels.

As many economically significant crops and animals, including humans, are susceptible to fungal and bacterial diseases, Finding cures for these diseases will need the

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methodical investigation of plant species in search of novel bioactive compounds. The value of plants comes from the chemical substances they contain, which have an observable impact on human physiology. The most well-known of these bioactive plant elements are alkaloids, tannins, flavonoids, and phenolic compounds. Low mammalian toxicity, target selectivity, and biodegradability are just a few of the ways in which these natural pesticides excel over their synthetic counterparts (Rajaiah et al., 2022).

Several primary and secondary metabolites as well as their intermediates, were found in the*Caesalpinia crista (Caesalpiniabonduc, Caesalpiniabonducella)* species of the familyCaesalpiniaceae (Fabeceae).They can potentially prevent and treat various disease conditions in humans (Panda et al., 2022). Its global reach included Southeast Asia and the rest of the tropics and subtropics. This plant can be found up to 2,500 feet above sea level in some of the woodlands along the coast and inland (Joshi et al., 2023).

Figure 1.*Caesalpinia crista* Plant

Bonducin (Bonducellin), Steroidal saponins, 1, 5, 6, 7, 14-Voucapanepentol derivative, Caesalpin, (1-ketone 6, 7-diacetylcassane), acetic acid, myristic acid, vinaticole, vouncapenic and cassaic acids, cassanefuranoditerpenoid as bonducellpin E, F, lupeol acetate, ß-amyrin and α-amyrin are reported in plant fruit cells (Dhulap et al., 2023; Almeleebia et al., 2022).

Mycobacterium tuberculosis is the primary pathogen responsible for tuberculosis (TB), a bacterial lung illness (*M. tuberculosis* [MTB]). Throughout the past few decades, it has been recognised as a serious problem since it is home to many of the world's most infectious and potentially fatal diseases. White plaque smear-positive tuberculosis is an infectious illness caused by Mycobacterium tuberculosis and related organisms. Several mycobacteria species include *M. africanum, M. bovis, M. caprae, M. microti, M. pinnipedii*and *M. canettii*(Wijaya et al., 2022).

People have different preferences for which *Caesalpinia crista* plants to use while treating disease symptoms. The goal of this research was, therefore, to determine whether or not *Caesalpinia crista* fruit contains any phytoconstituents with anti-tuberculosis properties.

Materials and Methods

Collections of Plant Material and Drying

Harvested in September, the ripe fruits of the plant are fresh and ready to eat. Raw medication is stored in the dark for regular room-temperature drying. Coarse powder in airtight containers was stored to avoid from direct sunlight and moisture until making the extract.

Authentication

Dr. Priyanka A. Ingle (Scientist, Botanical Survey of India, Pune) confirmed the authenticity of this plant specimen (Dated October 1, 2020, voucher specimen VVD 01 of *Caesalpinia crista* was submitted (Ref. No. BSI/WRC/IDEN.CER./2020/92)).

Crude Extract preparation

Two 12-hour sessions of Soxhlet apparatus refluxing with different organic solvents were applied to 100 grammes of dry powdered fruit components. Hexane, ethanol, chloroform, ethyl acetate, and distilled water were chosen as solvents for the extraction process due to their varying degrees of polarity. Keeping the solvent at a rolling boil throughout the extraction process is crucial for obtaining the highest possible yield. The extracted dried concentrate was then packaged in sterile containers, labelled, and stored in the fridge for later analysis.

Phyto-chemical Screening:

Caesalpinia crista fruit extracts were analysed for their physical properties and yield percentage after being concentrated and dried. In addition, a preliminary qualitative test was conducted on all extracts to check for primary and secondary phytochemicals, as is customary practice (Mukherjee, 2002; Harborne, 1998; Gokhale, 2020; Trease, 2002; Khandelwal, 2005; Wagner, 1996).

Phytochemical analysis of *Caesalpinia crista* crude extracts

Extracted phytochemicals may show a reaction in qualitative and quantitative phytochemical analysis. The phytochemicals in each extract were identified and quantified using the following techniques.

Determination of total phenolic compounds

The sample extract was diluted with 100 ml of distilled water after being weighed out at 100 mg. After removing 1 mL of the solution, 0.5 mL of 2N Folin-Ciocalteu reagent and 1.5 mL of 20% Na_2CO_3 solution were added to a test tube. The volume was then increased

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to 8 millilitres using distilled water, and the resulting mixture was vigorously shaken before being given two hours to settle. At 765 nm, we measured the absorption. The solubility of a 0.5 mg/ml standard gallic acid solution in methanol was determined using the same conditions. The results of every test were measured three times for accuracy (Silva et al., 2022).

Determination of total flavonoids

The move towards relies on the absorptivity maximum at 415nm, which is achieved through the production of the flavonoids-aluminium combination. Twenty percent aluminium trichloride in methanol was added to 100µl of sample extracts in methanol (10 milligrammes per millilitre). Add 0.1 millilitre acetic acid and a 5-millilitre methanol mixture. After waiting 40 minutes, the absorbance at 415 nm was measured. We followed the same laboratory protocols and created 100 cc of blank samples. Under the same laboratory conditions, quercetin absorption from a 0.5 mg/ml quercetin solution in methanol was tested as a reference. The results of every test were measured three times for accuracy (Donga et al., 2022).

Determination of total alkaloids

1 g of test extract was macerated in 20 ml of ethanol containing 20% sulfuric acid (1:1 v/v). 1 ml of the filtrate was mixed with 4 ml of the 60% H_2SO_4 solution. The aforesaid combination was left to stand for 3 hours after being combined with 5 ml of 0.5% formaldehyde in 60% H_2SO_4 for 5 minutes. At 565 nm, the absorbance was measured.

Determination of total tannins

3.75 ml of distilled water, 0.5 ml of 35% sodium carbonate solution, 0.25 ml of Folin Phenol reagent, and 0.5 millilitres of test extract was mixed carefully. The solution mentioned above was tested for its absorbance at 725 nm. Tannic acid standard solutions ranged from 0 to 0.5mg/ml. The concentration of tannic acid in the extract is reported as many milligram's per millilitre (Govindaram et al., 2023; Roghini et al., 2018).

Determination of total glycosides

We used 50 millilitres of distilled water to macerate 1 gramme of extract. Alkaline pirate solution (about 4 ml) was added to the filtrate (1 millilitre). After boiling the mixture for 5 minutes we let it cool down. Then, we measured the light absorbance of the mixture at a wavelength of 490 nm, as reported by Chen et al. (2022) and Mobin et al. (2021).

Test for Terpenoids

A 1 gramme sample was macerated in 50 ml of ethanol before being filtered. Combine 2.5 ml of filtrate with the same volume of concentrated hydrogen peroxide and 5% aqueous phosphomolybdic acid solution. Let for 30 minutes, then add enough ethanol to make up 12 ml. At 700 nm, we measured the absorption (Juvatkar et al., 2021).

Test for Steroids

A 1-gramme sample was macerated in 20 millilitres of ethanol before being filtered. A volume of 2 mL of the chromogenic solution was added into the filtrate and the solution was allowed to sit for thirty minutes. 550 nm was used to measure the absorbance. The concentration of individual phytocomponents in test extract can be estimated by measuring the colour difference between test and blank samples. All the quantitative information shown above is given in milligrams per dry material (Juvatkar et al., 2021).

Column chromatographic isolation

As per observation, ethanol and ethyl acetate extracts containing components were separated with isocratic manner. The *in-vitro* anti-tubercular activity of isolated fractions was tested (Bhanderi et al., 2022).

In-vitro Anti-Tubercular activity

Microplate Alamar Blue Assay (MABA)

Screening against mycobacterial pathogens (*Mycobacterium tuberculosis*) (H37Rv) (ATCC No-27294), employing 96 well plates in the microplateAlamar blue assay (MABA), is in the works.

Requirement

Microbial strain *M. tuberculosis* (H37Rv) (ATCC No-27294), Almar Blue reagent, Tween 80, parafilm, incubator, Middlebrook 7H9 culture media, and 96 well plates are all required.

Test sample: Several phytocomponents were detected in an ethanol extract of *Caesalpinia crista* fruit, and these components were separated using isocratic column chromatography. MicroplateAlamar Blue Assay assessed the concentration and quality of all collected fractions to identify the most active and purified phytocomponents. Preparations were made for test concentrations ranging from 0.8 to 100 µg/ml (Silva et al., 2022; Shirsat et al., 2021).

Microplate Alamar Blue Assay

M. tuberculosis (H37Rv) (ATCC No. 27294) was used to test the fractions' antitubercular activity. This approach strongly correlates with the proportionate and BACTEC radiometric methods and uses a thermally stable reagent. *M. tuberculosis* (MTB) was grown in 7H9 medium with and without the test plant components at dilutions of 0.8, 1.6, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml in a 96-well plate. To avoid medium evaporation during incubation 200 µl of sterile deionized water was added to the external wall wells of a sterile 96-well plate.

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Table 1. Characteristics of *Caesalpinia crista* fruit extracts.

Extract particulars	Percent Yield (%W/W)	Characteristics	
		Colour	Appearance/ Consistency
Hexane extract (CFH)	04.11%	Green	Solid
Chloroform extract (CFC)	08..31%	Brown	Semisolid
Ethyl acetate extract (CFEA)	14.25%	Dark brown	Semisolid
Ethanol extract (CFET)	10.25%	Dark brown	Semisolid
Aqueous (Water) extract (CFA)	07.67%	Brown	Liquid

Table 2. Qualitative analysis of *Caesalpinia crista* Fruits extract.

Sr.No.	Tests	CFH	CFC	CFEA	CFE	CFA
1.	**Tests for Acidic compounds**	-	-	-	+	-
2	**Test for carbohydrate**					
	Molish's test	-	-	-	+	-
	Fehling test	-	+	+	+	-
	Benedicts test	-	-	+	-	-
	Barfoed test	-	-	-	+	-
	Selivanoffs test	-	+	+	-	-
	Osazone formation test	-	-	-	-	-
3	**Test for Proteins**					
	Biuret Test	-	-	-	+	+
	Millons Test	-	-	-	-	-
4	**Test for amino acids**					
	Ninhydrine test	-	+	-	+	+
5	**Test for Steroids**					
	Salkowski test	+	+	-	-	-
	Libermann test	+	-	-	+	-
	Libermann-Burchard reaction	-	+	-	-	-
6	**Test for Glycosides**					
	Anthraquinone glycoside test	-	+	-	+	-
	Cardiac glycoside test	-	+	-	+	-
	Cynogentic glycosides test	-	-	+	-	-
7	**Test for Terpenoids:**	-	+	+	+	-

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8	**Test for Saponin**					
	Foam test	-	+	-	-	+
9	**Test for Alkaloids**					
	Dragondorff's test	-	+	+	+	-
	Mayer's test	-	-	-	-	-
	Hager's test	-	-	+	+	-
	Wagner's test	-	-	-	+	-
10	**Test for Tannins and Phenolic compounds**					
	5% $FeCl_3$ test	-	-	+	+	-
	Lead acetate solution	-	-	+	+	-
11	**Test for Flavonoids**					
	Shinoda test	-	-	+	+	-
	Sulphuric acid test	-	-	+	-	-
Abbreviations: HE- Hexane; CH- Chloroform; EA-Ethyl Acetate; EO; Ethanol; AQ; Aqueous, Note: (-): Absent, (+): Presence						

Table 3. Quantitative Analysis of *Caesalpinia crista* Fruits extracts

Extracts/ Test	Phytochemical Mean ± STD						
	Phenols	Flavonoids	Alkaloids	Tannins	Glycosides	Terpenoids	Steroids
***Caesalpinia crista* Fruits extracts**							
Hexane extract (CFH)	-	0.06 ± 0.003	-	-	0.77 ± 0.003	0.24 ± 0.021	1.23 ± 0.083
Chloroform extract CFCH	1.30 ± 0.030	-	4.65 ± 0.024	-	0.21 ± 0.002	1.31 ± 0.071	1.47 ± 0.012
Ethyl acetate extract (CFEA)	2.62 ± 0.011	5.58 ± 0.013	5.61 ± 0.042	1.68 ± 0.012	0.68 ± 0.001	4.08 ± 0.004	-
Ethanol extract (CFET)	4.05 ± 0.027	6.75 ± 0.019	4.70 ± 0.020	0.85 ± 0.011	1.75 ± 0.087	4.10 ± 0.052	-
Aqueous (Water) extract (CFAQ)	0.87± 0.021	-	-	0.16 ± 0.003	0.54 ± 0.020	-	-

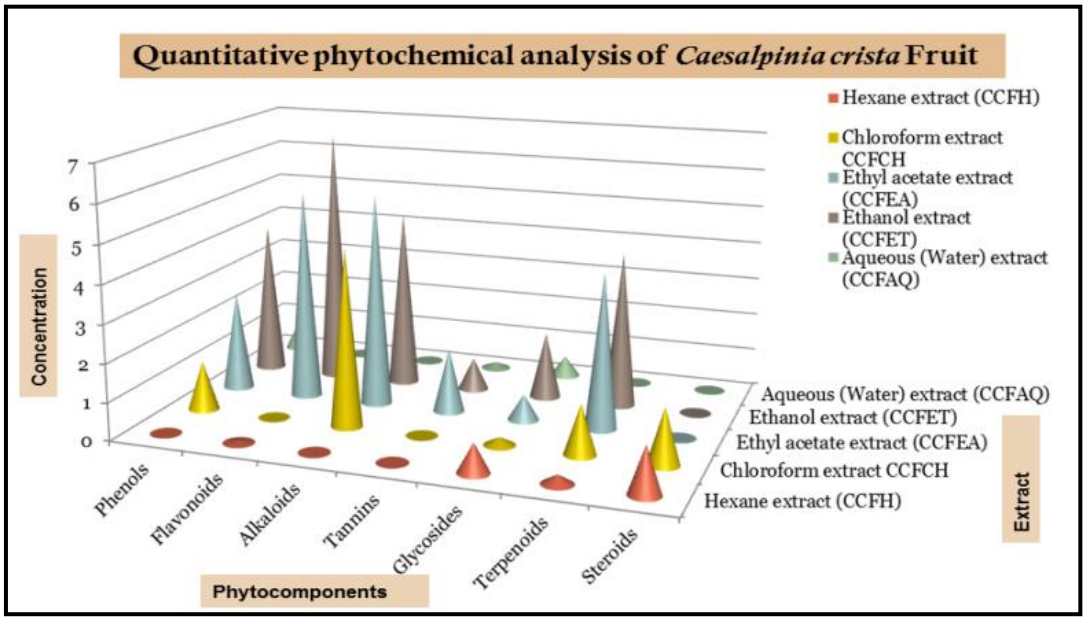

Figure 2.Concentration vs Test compound of *Caesalpinia crista* Fruits extract

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Each well of a plate was then filled with 100 µl of Middlebrook 7H9 broth, allowing for the serial dilution of compounds. The detected drug concentrations ranged from 0.80 to 100 g/ml.

Parafilm was used to seal the plates, and then they were incubated at 37 degrees Celsius for five days. The plate was then incubated for 24 hours after adding 25 µl of a freshly prepared 1:1 mixture of Almar Blue reagent and 10% Tween 80. Wells that tested blue indicated no bacterial growth, whereas those that tested pink indicated growth. The rate at which the dye was diluted was used as a proxy for bacterial proliferation. If the standard's result for the percentage reduction of Alamar blue dye was lower, then the extracts were declared effective. All assay variables were kept in triplicate wells. The lowest drug concentration that prevented the blue-to-pink colour change was established as the minimum inhibitory concentration (MIC) (Martolia et al., 2021; Bansal et al., 2020).

Results and Discussion

Phyto-chemical screening test

The following findings were reported from the detailed phytochemical analysis of *Caesalpinia crista* fruit extracts conducted in this study. The properties of each extract are listed in Table 1. The results showed the highest percentage yield for the extracts made using chloroform, ethyl acetate, and ethanol. The results for various phytochemicals in the extract showed promise. Table 2 shows that the different complex metabolites in the fruits of *Caesalpinia crista* were more clearly separated using the polarity gradient solvent selection. Hexane, chloroform, ethyl acetate, and ethanol extracts revealed steroids, saponins, glycosides, alkaloids, phenolic chemicals, and flavonoids, respectively. Proteins and saponins were extracted aqueously.

Caesalpinia crista's (Crude extract) phyto-chemical quantification

Seven phytochemicals (phenol, flavonoids, alkaloids, tannin, glycosides, terpenoids, and steroids) were screened for quantitatively in the extract was carried out using methods described in the literature, and the findings are presented in Table 3 and Figure no. 2. Alkaloids, phenolic compounds, flavonoids, and glycosides are some examples of secondary metabolites that contribute to a plant's potential for medicinal use. Phytochemical components in the extract were also quantified through additional analytical testing.

The ethanol extract found phenols, terpenoids, and flavonoids in the highest quantity. Ethyl acetate extract has the highest concentration of alkaloid and some trace flavonoids. The chloroform extract contained measurable amounts of alkaloids and steroids. It is shown that plant metabolites can be isolated under controlled circumstances.

In-vitro Anti-Mycobacterial activity

MicroplateAlamar Blue Assay (MABA)

Caesalpinia crista ethanol extracts showed the most promise in the tests, inhibiting the development of mycobacteria. Take note of what is shown in Figure 3. The best inhibition and MIC values came in at 3.2 and 6.25 µg/ml for tests 6 and 7, respectively.

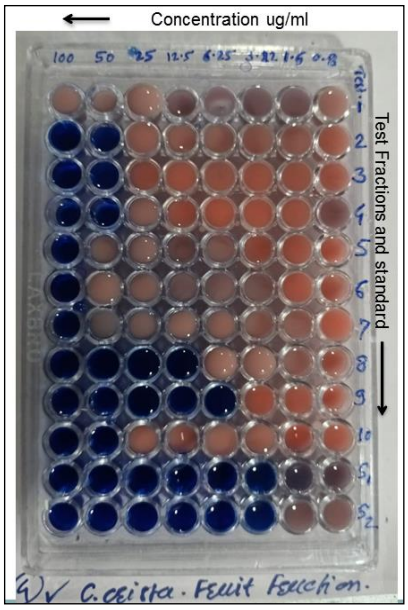

Figure 3.Image of Microplate Alamar Blue Assay (MABA)

Table 4. Minimum Inhibitory concentration (MIC) of specimens

Sr. No.	Samples	MIC µg/ml	Sr. No.	Samples	MIC µg/ml
1.	Fraction 1	100	7	Fraction 7	100
2.	Fraction 2	50	8	**Fraction 8**	**12.5**
3.	Fraction 3	50	9	**Fraction 9**	**6.25**
4.	Fraction 4	50	10	Fraction 10	50
5.	Fraction 5	100	11	Std. 1: Isoniazid	3.12
6.	Fraction 6	100	12	Std. 2: Rifampicin	3.12

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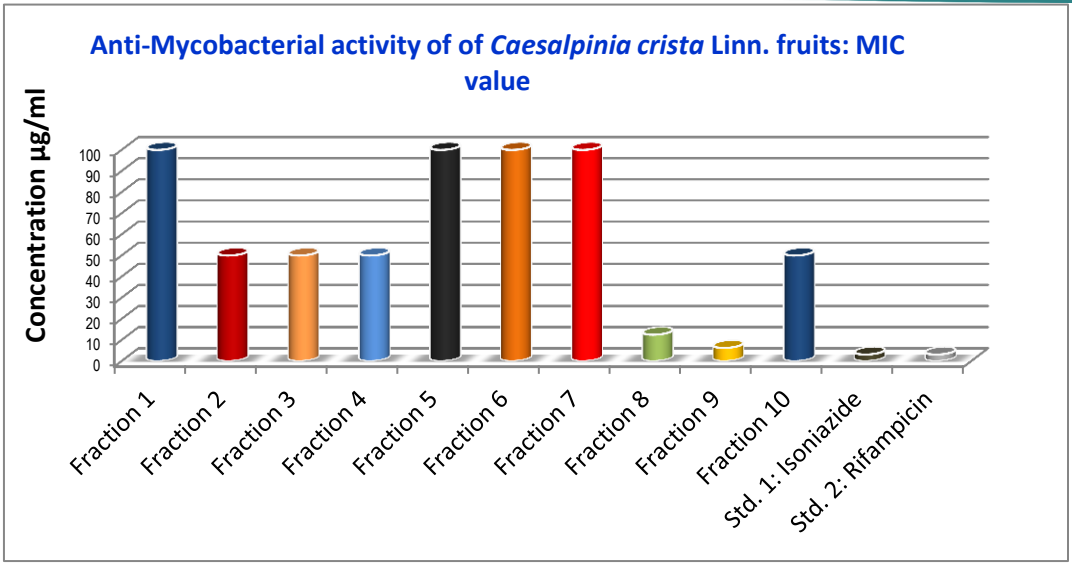

Figure 4.Graphical Illustration of Minimum Inhibitory concentrations vs fractions

The concentrations shown in Table 5 of the *in-vitro* anti-tubercular activity that results were effective in inhibiting the growth of *Mycobacterium.* Table 6 and Figure 2 reveal that the minimal inhibitory concentrations (MICs) for fractions 8 and 9 of the crude extract are 12.5 and 6.25 µg/ml, respectively. Hence, the separated phytochemicals in this fraction exhibited promising effectiveness against *M. tuberculosis*, especially when compared to other fractions and conventional medicines.

Conflict of Interest

There is no known conflict of interest in this publication.

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Conclusion

The fruits of *Caesalpinia crista* were extracted using a solvent and then tested for several phytochemicals. Qualitative analysis alongside sugars, proteins, and lipids also detected secondary metabolites like alkaloids, flavonoids, phenols, tannins, terpenoids, and glycosides. Some of the components necessary for biosynthesis that produces higher derivatives might be found in the fruits of plants. Alkaloids, flavonoids, phenols, tannins, terpenoids, and glycosides, among other phytocomponents, were abundant when the samples were analysed quantitatively. In particular, the ethyl acetate and ethanol extracts of Caesalpinia crista fruits had significantly more of this compound than the other three extracts. Alkaloids are necessary for substance metabolism as well as the protective function of animals. The use of flavonoids to treat malignant tumors is attributed to their capability to halt the growth and spread of malignant tumours. Numerous more disease manifestations have demonstrated the antioxidant ability of phenolic and flavonoid compounds. Several studies have found that combining phenols with plant flavonoid components increases the antioxidant, anticarcinogenic, anti-inflammatory, and other beneficial effects. The inclusion of tannins improves the antibacterial action of extracts, and they also inhibit pathogenic fungi.

Ethyl acetate extract fractions 8 and 9 were found to have identified phytochemical compounds with promising antimycobacterial activity. They have the potential to develop into a game-changing lead medication source for the prevention of tuberculosis due to their promising activity against the M. tuberculosis (H37Rv) strain. To sum up, *Caesalpinia crista*'s extracted bioactive fractions and phytocomponents are promising candidates for use as antimycobacterial medicines in future pharmaceutical and light-emitting diode (LED) innovations. We need to better understand the active portions of ethyl acetate and isolate the functional groups responsible for its antibacterial effects.

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