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PHYTOCHEMICALS ANALYSIS VIA GC-MS, ASSESSMENT OF ANTI-INFLAMMATORY, ANTI-BACTERIAL, AND ANTI-FUNGAL PROPERTIES OF *CASSIA FISTULA* POD-BASED FUNCTIONAL TEA

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Abstract

Cassia fistula Linn, also known as Pakistan Amaltas and Indian Laburnum, is a tropical plant used in traditional medicine for treating syphilis, tumors, burns, constipation, and skin disorders. The study aimed to ascertain the *Cassia fistula* Linn pods' zone of inhibition against bacterial strains and inflammation, in addition to assessing the pods' potential as antibacterial and ant-inflammatory agents. The hydroalcohol extracts' possible antibacterial efficacy against important bacterial and fungal species for medicine was evaluated. The antibacterial activity of *Cassia fistula*'s pod extracts (5, 25, 50, 100, and 250 µg/ml) against a variety of pathogenic bacteria and fungal species was

assessed using the agar disc diffusion method. The extracts showed a significant decrease of inflammation and bacterial growth against the tested species. The microbiological activity of *Cassia fistula*'s pods has been associated with a number of secondary metabolites. The identification of naturally occurring bioactive chemicals in these plants may open the door to new directions in pharmacological research. The zone of inhibition was compared to a number of standards, such as ampicillin, ciprofloxacin, norfloxacin, chloramphenicol, nystatin, and griseofulvin. Anti-inflammatory teat showed a typical immunological response in LPS-stimulated cells, with TNF- and NO levels of 85.9% and 76%, respectively. There is more potential for hydrocortisone and *Cassia fistula*'s pods to reduce particular inflammatory markers. The antibacterial test showed growth inhibition zones against S. pyogenes, S. aureus, E. coli, and P. aeruginosa at dosages ranging from 5 to 25 µg/ml. The amount of tannin, alkaloids, saponin, and total phenolic content were all reduced by the *Cassia fistula*'s pod extraction. Immunomodulatory investigations revealed a greater blood antibody titer in the extract-fed group. The GC-MC technique was utilized to analyze phytochemical compounds in the Cassia fistula pod extract, revealing thymine, butanoic acid, 2-methyl-, 2-methylpropyl ester, furancarboxaldehyde, pentanoic acid, 1,1 dimethylethyl ester, 5-Acetoxymethyl-2-furaldehyde, butanoic acid, Valeric acid, 2,4;3,5-Dimethylene-1-iditol, Vitamin E, n-Hexadecanoic acid, Myo-Inositol, 4-C-methyl, oleic acid, and a-sitosterol.

Keywords: *Cassia Fistula* (Amaltas), Phytochemical Compounds, pods, Anti-inflammatory activity, Anti-bacterial activity, Anti- fungal activity, Functional tea.

Introduction

Cassia fistula is a member of the Leguminosae family and is also referred to as Indian Laburnum or Amaltas, A semi-wild plant having therapeutic qualities. Asia, South Africa, China, the West Indies, and Brazil are among the regions where it is available (Verpoorte *et al*., 1999). The plant, which grows throughout India's deciduous and mixed monsoon forests, is widely utilised in the Ayurvedic system to treat a wide range of illnesses. In the outer Himalaya, it can reach heights of up to 1300 meters (Joshi *et al*., 2004). Phytochemicals are often present in medicinal plants. According to reports, phytochemical compounds that are commonly used to combat cancer, diabetes, fungus, bacteria, and inflammation. In recent years, phytochemicals including the phenolic compounds present in many herbs have drawn a lot of interest for their potential health advantages, such as their antioxidant and anti-inflammatory properties. Additionally, interest in using herbs as functional components in meals has grown among processors and consumers as a result of their well-known culinary characteristics. The plant can be found up to 1300 metres in the outer Himalayas of India, where it can be found in deciduous and mixed monsoon forests. In Maharashtra, it is grown as an ornamental plant (Batna and Balaraman, 2005). It has been discovered that *Cassia fistula* helps with skin conditions, liver issues, tuberculose glands, hematemesis, pruritus, leucoderma, and diabetes. Because of its high fibre and mucilage content, it may be used as a hypercholesterolemia treatment agent. In India, the plant extract is also suggested for the management of illnesses and pests. According to Alam *et al*. (1990), its leaves are used to treat inflammation, boils, ulcers, and skin conditions. Living mammals' localised inflammatory response to damage is a form of self-defense. Interest in novel medications possessing this characteristic has surged due to the advent of non-steroidal treatments for human ailments such as rheumatoid arthritis (Anonymous, 2005). Interest in novel medications with this characteristic has increased as non-steroidal treatments for human conditions including rheumatoid arthritis have emerged. Acute inflammation is brought on by the blood's leukocytes and plasma entering wounded tissues. Inflammation can be either acute or chronic. The local vascular system, the immune system, and the cells within the wounded tissue are all involved in this biochemical chain reaction that results in this response. Pain, Redness, Immobility, Swelling, and Heat are the five cardinal indicators of inflammation that are identified by the acronym "PRISH" (Kokashi, 1958). Drug-resistant bacteria and hazardous reactions pose a danger to the efficiency of antibiotics, which are essential in the fight against bacterial diseases. We need newer medications that are less resistive (Farnsworth, 1993). In underdeveloped nations, natural remedies like traditional medicine are essential for both disease

prevention and treatment (Houghton, 1995). Traditional medicine makes extensive use of herbs; in fact, between 1981 and 2002, natural items were the basis for 61% of newly created pharmaceuticals. On the other hand, fewer unique chemical entities are being discovered on a regular basis. According to Ramasamy (2009), higher plant natural compounds might provide fresh antibacterial agents with innovative processes. Plants are useful in traditional medicine because they contain secondary metabolites that have antibacterial qualities. According to estimates from the World Health Organization, 80% of people on the planet receive traditional medical care using plant extracts. But bacteria that are resistant to drugs have surfaced, endangering diseases. According to Towers *et al*., (2001), bacteria possess the genetic capacity to transfer and develop resistance to synthetic medications, which has led to an increase in resistance to novel antibiotics. In the current study, phytochemicals compounds were quantified in the extracts of *Cassia* fistula's pods based functional tea using the gas chromatography-mass chromatography (GC-MC) technique. The new sources of anti-inflammatory and anti-bacterial and anti-fungal agents are found by screening various extracts of *Cassia fistula* pod-based functional tea against pathogenic bacteria, fungi, and inflammation.

Material and Method

The study at Bahauddin Zakariya University in Multan, Pakistan, involved gathering Cassia fistula's pods from Punjab and drying them to create a powder, using analytical grade chemicals and following standard laboratory procedures.

1. Samples Collection

The study collected Cassia fistula pods from Multan, Pakistan, including the university's Bio-Park. The samples were named, stored in polythene bags, and sent to the Botany department for identification. The study used only the pod portion of the Cassia fistula, and the materials were stored at room temperature.

2. Phytochemical Compounds Analysis via GC-MS Analysis

Using the GC-MC technique, the phytochemical components in the extract of *Cassia fistula*'s pods were measured using the Hussain *et al*., (2009) method. Gas chromatography mass spectrometry (GC-MS) is a scientific technique that merges the benefits of mass spectrometry and gas-liquid chromatography for the identification of different compounds within a sample. The primary goal of this analytical method is to determine the quantity of a substance by comparing the relative concentrations of atomic masses in the generated spectrum. Both comparative and innovative analyses are options. Comparative analysis basically examines a spectrum to check whether any of its properties are found in a sample of a library of spectrums. Because of the numerous visual distortions that might occur as a result of scale differences, a computer is the ideal tool for performing this. To more precisely relate certain data, computers can concurrently correlate other data (such the retention durations found by GC). A different analytical technique compares the peaks to one another. According to this system, the tallest peak receives a value of 100%, while the remaining conferences receive proportionate values. All values over 3% have a given value. Typically, the parent peak represents the whole mass of the unidentified chemical. The value of this parent peak can be used to fit a chemical equation that includes all of the components that are thought to be present in the molecule. It is possible to distinguish the different elements present by looking at the isotope pattern in the spectrum, which is particular to elements with several isotopes. The molecular structure and bonding may be determined once a chemical formula has been matched to the spectrum, and they must be compatible with the traits noted by GC-MS. Typically, given a list of the constituents that may be present in the sample, this identification is carried out automatically by programmes that come with the instrument.

Preparation of Extract

For the GC/MS analysis, 2 L of the ethanolic extract of *Cassia fistula* were used.

Instruments and Chromatographic Conditions

For the GC-MS analysis, we utilized a GC Clarus 500 Perkin Elmer system equipped with an AOC-20i autosampler, and it was interfaced with a gas chromatograph and mass spectrometer apparatus. The analysis was performed under the following conditions:

GC Programme

Column

- Equipment: GC Clarus 500 Perkin Elmer
- Elite-5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30 0.25 mm 0.25 m df;
- Carrier gas: 1 ml per min, Split: 10:1
- Detector: Turbo mass gold Perkin Elmer mass detector
- 5.2 Turbo mass software
- 2 µl of sample was injected.

Oven Temperature Programme

- Initial temperature of 110 °C held for 2 minutes.
- Temperature increased at a rate of 10°C per minute until reaching 200 °C, with no holding period.
- Temperature further increased at a rate of 5°C per minute until reaching 280 °C, with a 9-minute hold.
- Injector maintained at a temperature of 250°C throughout the analysis.
- The total duration of the gas chromatography run is 36 minutes.

MS Programme

- Utilized library: NIST Version-Year 2005
- Inlet line temperature: 200 ℃
- Source temperature: 200 ℃
- Electron energy: 70 electronvolts (eV)
- Mass scan range (m/z): 45 to 450
- Solvent delay: 0 to 2 minutes
- Overall duration of the mass spectrometry analysis: 36 minutes

3. Anti-Inflammatory Activity

Examination of anti-inflammatory medications The *Cassia fistula* pods were investigated use the method of Anitha and Miruthula (2020). One milliliter (ml) of lipopolysaccharide (LPS) was added to each well of a 96-well plate after one x 105 WBCs had been seeded there to cause inflammation. After being incubated in a CO_2 incubator for 24 hours, the cells were treated for 72 hours with 100 µL of medium, different extracts, or conventional hydrocortisone at escalating doses. Then, using the same process as previously mentioned, the MTT solution was added to each well. The stimulation index (SI) was computed in order to get the effective concentration (EC) for each extract and hydrocortisone. SI is the absorbance obtained from hydrocortisone- or extract-treated LPS-stimulated WBCs divided by the absorbance of untreated WBCs. The concentration needed to return LPS-stimulated cells with an aberrant SI value to their normal condition ($SI \approx 1$) is represented by the EC.

4. Anti-bacterial and Anti-fungal Assay

Pods of *Cassia fistula* were subjected to antibacterial activity tests as outlined in Bhalodia and Shukla (2021) and Duraipandiyan and Ignacimuthu (2017). Zone of inhibition method determination. The in vitro bactericidal activities of hydro alcohol extracts were studied. The agar disc diffusion technique was used to assess the antibacterial activity of pod extracts against four pathogenic bacteria (both Gram-positive and Gram-negative) and three pathogenic fungi. The agar cup technique was used to evaluate the antibacterial activity. Every single pure extract was first denatured by dissolving it in dimethyl sulfoxide, then sterilised by filtering it through a fritted glass filter disc, and finally kept cold at 4°C. All of the extracts were evaluated for their antibacterial activity against Pseudomonas

aeruginosa, Escherichia coli, Staphylococcus aureus, and Streptococcus pyogenes. Sets of five dilutions (5, 25, 50, 100, and 250 g/ml) of the pod extract and conventional medications were prepared using nutrient agar tubes and double-distilled water. In order to evaluate the antibacterial activity, sterile Mueller-Hinton agar plates were first used to disseminate indicator bacterial strains (108 cfu), which were then incubated for three hours at 37°C. To assess their antibacterial effectiveness, ampicillin, chloramphenicol, ciprofloxacin, and norfloxacin were used as controls under comparable circumstances. After incubating at 37°C for 18–24 hours for bacteria and 48–96 hours for fungus, the regions around the discs that showed growth inhibition were investigated. By measuring the widths of the inhibitory zones, including the disc diameter, on the agar, the sensitivity of the microorganism species to the pod extracts was ascertained.

Results and Discussion

1. Identification of Phytochemical via GC-MS

The chromatographic analysis of the ethanol extract derived from *Cassia fistula* revealed the presence of thirteen distinct peaks, indicating the existence of thirteen unique phytochemical constituents such as Thymine, Butanoic acid, 2-methyl-, 2-methylpropyl ester, 2 furancarboxaldehyde, 5 (Hydoxymethyl), Pentanoic acid, 1,1 dimethylethyl ester, 5-Acetoxymethyl-2-furaldehyde, Butanoic acid, 2-methyl-, 2-methylpropyl ester, Valeric acid, 4-tridecyl ester, 2,4;3,5-Dimethylene-1-iditol, Vitamin E, n-Hexadecanoic acid, Myo-Inositol, 4-C-methyl, Oleic acid and a-sitosterol. These thirteen phytochemical compounds were identified and characterized by cross-referencing their mass spectra with the information available in Dr. Duke's Phytochemical and Ethanol botanical databases. It was demonstrated how the plant's numerous phytochemicals contribute to its therapeutic properties. The mass spectra of each phytochemical found in the methanolic extract of the *Cassia fistula*'s pods plant shown in the table no. 1.

Table no. 1: Component Identified in the *Cassia fistula*'s Pods (GC-MS Study).

Sr #	RT	Name of Phytochemical Compound	MF	MW	PA%
1	3.07	Thymine	$C_5H_6N_2O$	126	1.16
2	4.12	Butanoic acid, 2-methyl-, 2-methylpropyl ester	$C_9H_{18}O_2$	158	0.77
3	4.88	2-furancarboxaldehyde, 5 (Hydoxymethyl)	$C_6H_6O_3$	126	5.28
4	7.39	Pentanoic acid, 1,1 dimethylethyl ester	$C_9H_{18}O_2$	158	1.28
5	7.97	5-Acetoxymethyl-2-furaldehyde	C8H8O4	168	1.80
6	8.70	Butanoic acid, 2-methyl-, 2-methylpropyl ester	$C_9H_{18}O_2$	158	0.77
7	10.59	Valeric acid, 4-tridecyl ester	$C_{18}H_36O_2$	284	0.97
8	11.67	2,4;3,5-Dimethylene-1-iditol	$C_{18}H_{14}O_6$	206	12.76
9	12.8	Vitamin E	$C_{29}H_{50}O_2$	430	0.27
10	12.76	n-Hexadecanoic acid	$C1_6H_{32}O_2$	256	7.35
11	13.88	Myo-Inositol, 4-C-methyl	$C_7H_{14}O_6$	194	64.82
12	14.92	Oleic acid	$C_{18}H_{34}O_2$	282	1.5
13	31.19	a-sitosterol	$C_{29}H_{50}O$	414	0.75

Retention Time (RT), Molecular Weight (MW), Molecular Formula (MF) and Peak Area (PA) are all capitalized

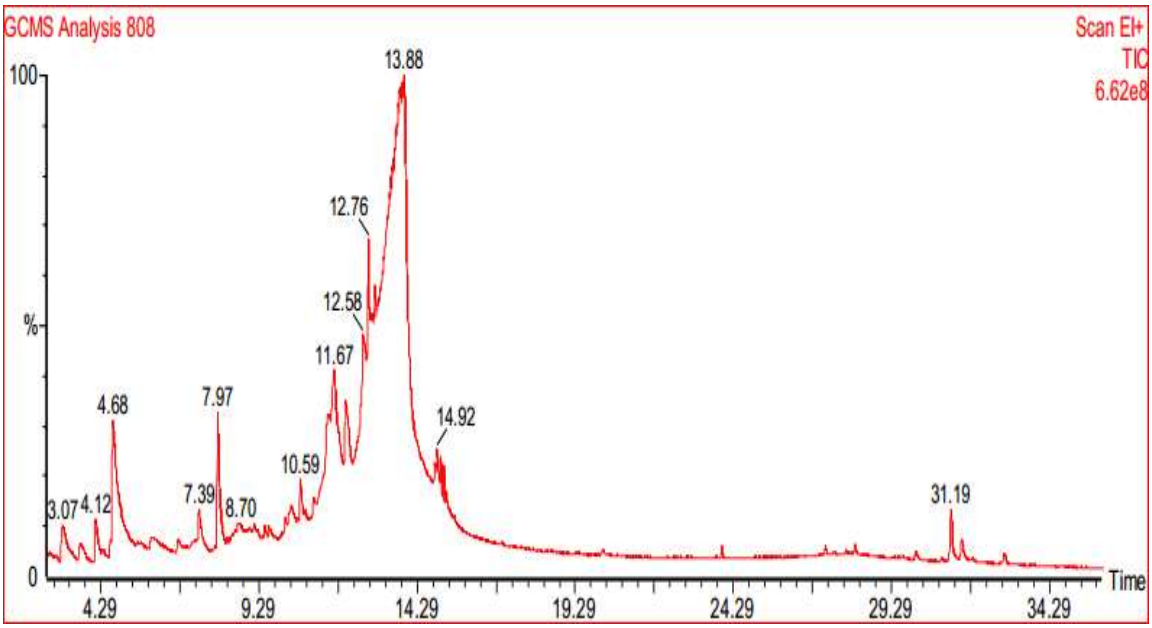

Figure no. 1: GC-MS Chromatogram – *Cassia Fistula*'s pods.

2. Anti-Inflammatory Activity

The *Cassia fistula*'s pod extracts showed high safety in restoring the abnormal stimulation index of LPS-stimulated cells WBCs into a normal immunological response shown in Table No. 2. The extracts effectively lowered inflammatory markers NO and TNF, with a significant reduction in TNF- and NO levels. The combination of hydrocortisone and *Cassia fistula* extracts also inhibited the expression of targeted inflammatory markers. The extracts also inhibited NF-kB activity, which can affect NO regulation. The GC-MS analysis identified compounds with anti-inflammatory properties, including tannins, anthraquinones, alkaloids, and flavonoids. Studies have shown that reactive oxygen species and nitric oxide are released when phagocytes invade inflammatory areas (Lim *et al*., 2001), leading to tissue injury. *Cassia fistula*'s pods contain alkaloids, tannins, flavonoids, terpenes, sugars, and glucosides, which have been linked to anti-inflammatory, antinociceptive, and antioxidant pathways (El-Meligy *et al*., 2015). The results of the GC-MS analysis suggest that *Cassia fistula* has a higher amount of anti-inflammatory action. The data presented in tables' no. 2, 3, and 4 collectively indicate that a combination of hydrocortisone and an extract from the pods of *Cassia fistula* has a greater ability to inhibit the expression of the targeted inflammatory markers. Previous studies have shown that the extracts from *Cassia fistula*'s pods can inhibit NF-kB activity, which in turn stops pro-inflammatory mediators like p38, JNK, and ERK1/2 from being activated by mitogen-activated protein kinases. Because NF-kB inhibiting suppressed TNF, COX-2 expression may have an effect on NO regulation.

Table no. 2: Determination of the combination and EC100 toxic/anti-inflammatory dosage of extracts from the pods of Cassia fistula on human WBCs.

Component	Safe dose EC100(µg/ml)	Effective dose EC (µg/ml)
St. drug (Hydrocortisone)	102.37±4.1	58.32±5.12
Cassia fistula's pods	747.0.8±9.4	41.67±2.14
Mix	922.61±2.1	76.396±1.27

*****The data is presented in the form of a mean ±S.D and EC; Effective concentration.

Table no. 3: Comparison of extracts and conventional drug-treated WBCs stimulated with LPS vs LPS-induced WBCs and untreated control WBCs regarding TNF-α (pg/ml) and NO (nmol/ml) levels.

Component	TNF-α(pg/ml)	NO (nmol/ml)
-ve Control (untreated normal cells)	41.87±1.2	28.84±0.5
St. drug (Hydrocortisone)	77.22±1.5	62.94±2.3
Induced	295.42±4.6	99.48±5.4
Cassia fistula's pods	47.90±1.1	36.62±3.7
Mix	41.87±1.0	24.42±0.6

The data is presented in the form of a mean ±S.D

Table no. 4: Differences in the degree of COX-2 expression between WBCs activated by LPS, treated with extracts, and given a standard medication, compared to WBCs stimulated by LPS on its own

Component	COX-2
-ve Control (untreated normal cells)	0.0007±0.00005
St. drug (Hydrocortisone)	0.034±0.00084
Cassia fistula's pods	0.045±0.0047
Mix	0.00091±0.000008

*****The data is presented in the form of a mean ±S.D

3. Anti-bacterial and Anti-fungal Assay

Cassia fistula, a plant with various parts, has been found to possess strong antibacterial properties against various germ species. Four pathogenic bacterial strains, two Gram-positive and two Gram-negative, were used to assess the antimicrobial efficacy of *Cassia fistula*'s pod extracts. The results showed that the extracts were more effective against S. pyogenes and S. aureus than E. coli and P. aeruginosa. The study also revealed that hydro alcohol extracts from *Cassia fistula*'s pods have strong antibacterial qualities. The phytochemical research revealed that most plant constituents, including proteins, amino acids, glycosides, anthraquinone, flavonoids, triterpenoids, and steroids, have biological activity and may have medicinal benefits. The hydro alcohol extract was selected for the study due to its higher yield of chemical components. The results validate the application of pods in traditional medicine to treat bacterially-induced infectious illnesses. Further investigation is needed to determine the effectiveness of crude extracts as antibacterial agents and to isolate and identify the structure of the plant's potent antibacterial ingredients.

Table no. 5: Anti-microbial activity of *Cassia fistula*'s pods

The antibacterial properties of hydro alcoholic extracts derived from *Cassia fistula*'s pods					
Antibacterial characteristics (Inhibition Zone)					
Microorganism	***Cassia fistula* - Zone of inhibition (mm)**				
	Concentration (µg/ml)				
	Hydro alcohol extracts (µg/ml)				
	5	**25**	**50**	**100**	**250**
E. coli	0	14	17	18	21
P. aeruginosa	0	11	16	17	21
S. aureus	0	10	14	16	19
S. pyogenes	0	12	16	17	19

*****The data is presented in the form of a mean ±S.D

Table no. 6 shows the effects of popular drugs on bacterial strains E. coli, P. aeruginosa, S. aureus, and S. pyogenes. Ampicillin, chloramphenicol, ciprofloxacin, and norfloxacin showed superior

potential against these strains. However, at 50 and 100 µg/ml concentrations, these drugs had limited impact. The results indicate that *Cassia fistula*'s pod extract can influence these bacteria (E. coli, P. aeruginosa, S. aureus, and S. pyogenes) Its results demonstrated that extracts of *Cassia fistula*'s pods have potential anti-bacterial activity against the bacteria and fungus strains.

Table no. 6: The antibacterial effectiveness of standard drugs against a bacterial test organism.

Zone of Inhibition in mm					
Drug	**Concentration**	**Antibacterial properties**			
		E. coli	***P. aeruginosa***	***S. aureus***	***S. Pyogenes***
Ampicillin	5	15	15	12	11
	25	16	16	15	13
	50	17	16	17	14
	100	20	19	19	16
	250	21	21	20	19
Chloramphenicol	5	15	15	11	13
	25	18	18	14	15
	50	24	20	21	21
	100	24	20	21	21
	250	24	22	21	22
Ciprofloxacin	5	21	21	17	18
	25	24	24	20	20
	50	29	25	23	23
	100	29	27	22	23
	250	29	28	23	23
Norfloxacin	5	23	19	19	20
	25	26	20	20	21
	50	27	22	21	22
	100	18	24	22	27
	250	30	24	22	29

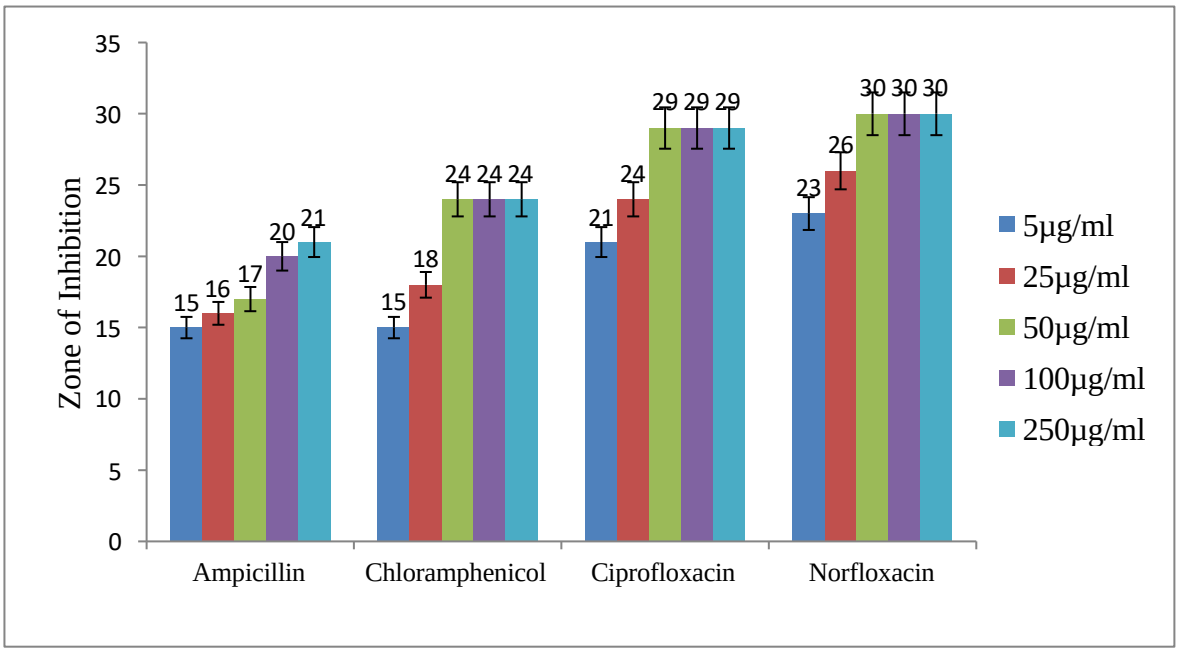

Figure No. 2: Anti-bacterial activity against *E. coli* (MTCC 443).

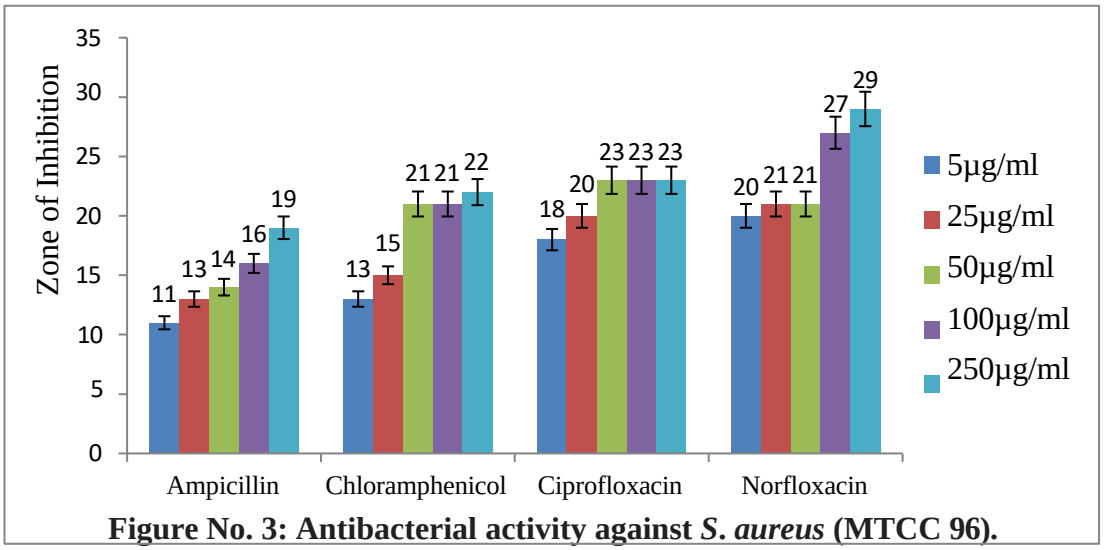

Figure No. 3: Antibacterial activity against *S. aureus* (MTCC 96).

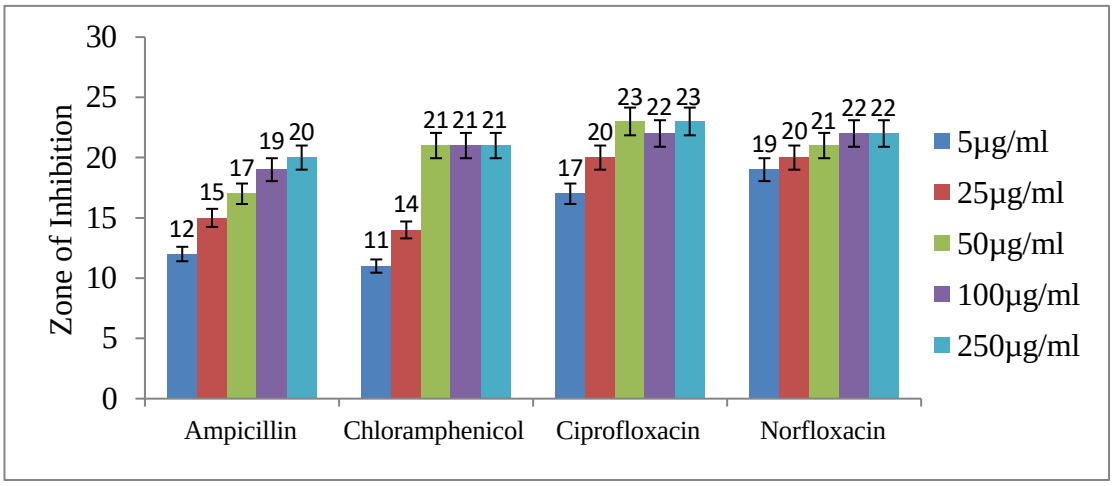

Figure No. 4: Anti-bacterial activity against *P. aeruginosa* (MTCC 424).

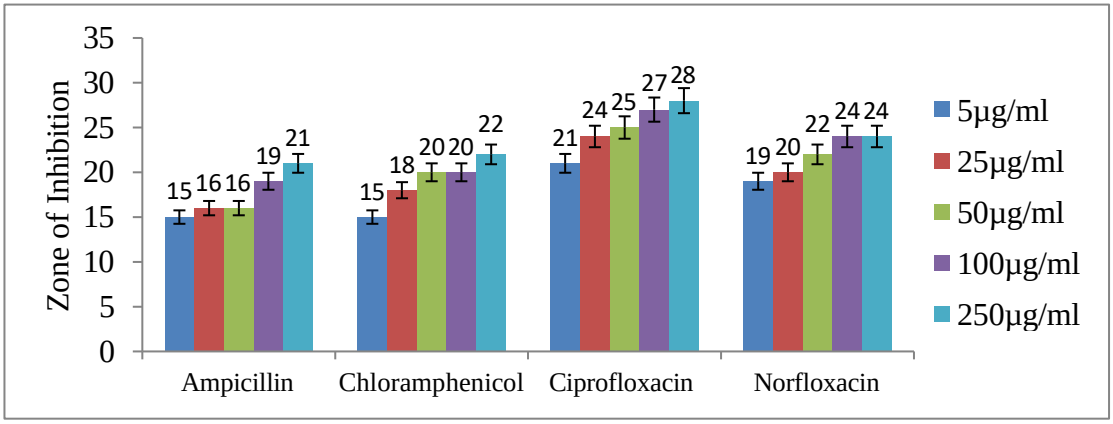

Figure No. 5: Anti-bacterial activity against *S. pyogenes* (MTCC 442).

Conclusion

The study on *Cassia fistula*'s pods found that its hydroalcohol extract is more effective in treating microbially-induced infectious illnesses than water or methanol extracts. The extract is more suitable for clinical research and is effective against most clinically isolated fungus and bacteria. The findings validate the medical application of pods, but further investigation is needed to assess the potency of crude extracts as antibacterial agents. The study also assessed *Cassia fistula*'s anti-inflammatory properties using phytochemical research, including tannin, anthraquinone, alkaloids, and flavonoids.

In-vitro experiments are planned to address ethical concerns and animal use in experimental pharmacological research.

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Author's Contributions
The writers of the original manuscript, Muhammad Naeem Zubairi, Muhammad Khurram Afzal, Ahmad Mujtaba Noman and Saeed Akhtar, Ali Musarrat were responsible for conceptualization, evaluating, and editing. Nayab Rao, Wisha Saeed and Mavra Ameen handled the formal analysis, research, funding procurement, reviewing, and editing. Resources, Muhammad Tauseef Sultan, Muhammad Usman Khalid, Hafiz Muhammad Fayyaz, and data curation and oversight

Declaration of Conflicting Interests
There are no possible conflicts of interest that the authors have disclosed about the research, writing, or publication of this article.

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Ethical Approval
Bahauddin Zakariya University in Multan, Pakistan 196/PEC/2022 has authorized the current study. Notably, all animal experiments was carried out with consideration for the welfare of the animals and with the least amount of potential harm, in compliance with all applicable laws, regulations, and standards.

Data Availability
Data will be provided at the corresponding author's reasonable request.

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