Trop J Nat Prod Res, November 2025; 9(11): 5464 - 5470

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Evaluation of the Bioactive Potential of *Cassia fistula* Stem Bark Exhibiting Antioxidant, Antimicrobial, Anti-inflammatory, and Cytotoxic Activities

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ARTICLE INFO

Article history:
Received 19 May 2025
Revised 06 November 2025
Accepted 12 November 2025
Published online 01 December 2025

ABSTRACT

Cassia fistula is a medicinal plant known for its wide range of traditional applications. The present study evaluated the bioactive potential of *C. fistula* stem bark (CFSB) extracts through antioxidant, antimicrobial, anti-inflammatory, and cytotoxic assays. Sequential extraction with n-hexane (CF-1), chloroform (CF-2), ethyl acetate (CF-3), and methanol (CF-4) was performed to yield distinct crude fractions. Antioxidant properties, total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), cytotoxic potential, antimicrobial, and *in vitro* anti-inflammatory activities of the extracts were determined. The CF-3 displayed significant antioxidant activity (IC_{50} = 8.45 µg/mL), close to standard ascorbic acid (IC_{50} = 6.54 µg/mL), aligning with TAC results. Also, it demonstrated higher TPC and TFC values, emphasizing its robust antioxidant potential. Cytotoxicity analysis revealed moderate activity in the CF-1 (LC_{50} = 30.70 µg/mL) compared to vincristine sulfate (LC_{50} = 3.50 µg/mL). The antimicrobial assay showed that CF-2 and CF-3 exhibited mild to moderate inhibition against Gram-positive bacteria (*Bacillus megaterium* and *Staphylococcus aureus*) and the CF-3 was also active against Gram-negative bacteria (*Salmonella typhi,* and *Escherichia coli*), but CF-2 was inactive against these strains. Mild antifungal activity against *Aspergillus niger* was observed for CF-2 and CF-3, while all extracts were inactive against *Aspergillus flavus*. *In vitro* protein denaturation studies demonstrated moderate anti-inflammatory activity by CF-4, comparable to diclofenac sodium. These findings suggest that different extracts of CFSB hold promise sources of bioactive compounds with antioxidant, antimicrobial, and anti-inflammatory potential. Further studies may facilitate the isolation of these compounds for potential therapeutic applications.

Keywords: *Cassia fistula*, Antioxidant, Phenolic content, Flavonoid content, Cytotoxicity, Antimicrobial, Anti-inflammatory.

Introduction

Medicinal plants are crucial sources of bioactive compounds with diverse therapeutic potential. Historical evidence supports the longstanding use of plant-based remedies in traditional medicinal practices, like Ayurveda, Unani, and Chinese traditional medicine, where herbs were employed for disease treatment and to restore bodily functions.[1-3] These traditional practices have significantly influenced modern therapeutic developments. Approximately 40% of today's pharmaceuticals are derived from natural sources, exemplified by breakthrough drugs like aspirin, artemisinin, and treatments for childhood cancers, all rooted in traditional knowledge.[4] The World Health Organization (WHO) estimated that 80% of the population of some Asian and African countries rely on traditional medicine for basic health care provision, with medicinal plant use increasing even among younger populations.[5]

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Citation: Paul R, Nishan TA, Uddin MF, Jhumur NT, Das AK, Saha K. Evaluation of the Bioactive Potential of *Cassia fistula* Stem Bark Exhibiting Antioxidant, Antimicrobial, Anti-inflammatory, and Cytotoxic Activities. Trop J Nat Prod Res. 2025; 9(11): 5464 – 5470 https://doi.org/10.26538/tjnpr/v9i11.29

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The therapeutic potentials of plants are largely dependent on their bioactive phytochemicals, which exhibit antioxidant, antimicrobial, and anti-inflammatory effects.[6] Many plants are abundant in antioxidants, such as polyphenols, that play critical roles in mitigating free radical activity, neutralizing reactive oxygen species, and decomposing peroxides. These antioxidant properties are linked to reduced risk and mortality from various diseases.[7] Although synthetic antioxidants are commonly used in the food industry, concerns over their toxicity have fueled the search for safer, natural alternatives.[8] Plant-derived antioxidants often demonstrate cytotoxic effects against cancer cells, highlighting their potential as both therapeutic and preventive agents. Compounds like curcumin, from turmeric, and quercetin, from fruits and vegetables, exemplify this association between antioxidant and cytotoxic activities, possessing strong free radical-scavenging and anticancer properties.[9] Evaluating the cytotoxicity of plant extracts is essential to determine their potential for commercial therapeutic applications.

The prevalent and unsanctioned utilization of antibiotics has led to increasing antimicrobial resistance, a major public health concern highlighted by the WHO. This trend underscores the need to develop alternative antimicrobial agents to mitigate antibiotic resistance. Consequently, research has focused on isolating new bioactive compounds from plants with potential antimicrobial properties.[10] Similarly, conventional anti-inflammatory drugs, though effective, often cause adverse effects, leading researchers to explore natural anti-inflammatory agents based on ethnopharmacological knowledge.[11] *Cassia fistula* Linn., generally recognized as the Golden Shower tree, belongs to the Caesalpiniaceae family and has historically been utilized for its medicinal significance. Known for its pharmacological properties, *C. fistula* has been used to treat ailments ranging from asthma and skin conditions to cardiovascular diseases and infections.[12] It exhibits diverse biological activities, including antimicrobial,[13-15]anti-

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

inflammatory,[16,17] antioxidant,[18-20] antitumor,[21] hypoglycemic,[22] and anti-diabetic,[23] effects. Various plant parts, including bark, leaves, roots, fruit and seeds, are utilized for various treatments (details in supplementary materials, Table S1). For example, stem bark and fruit extracts are known for their detoxifying potentials, with the seeds being utilized to manage gastrointestinal issues and as insect repellents.[24] While research has extensively covered various parts of *C. fistula,* studies on its stem bark, a component frequently used in traditional medicine, remain limited.

Therefore, this research aimed to fill this gap by comprehensively evaluating the antioxidant capacity, cytotoxicity, antimicrobial, and anti-inflammatory activities of *C. fistula* (CFSB) extracts, thereby advancing the understanding of its therapeutic potential. The research presents a comprehensive analysis of the bioactive value of CFSB, an area not previously explored in scientific literature despite its use in traditional medicine. Unlike prior studies focusing on leaves, fruits, or roots, this research systematically investigates the antioxidant, antimicrobial, anti-inflammatory, and cytotoxic properties of sequential solvent extracts (n-hexane, chloroform, ethyl acetate, and methanol). The study establishes a strong linkage between the levels of phenolic and flavonoid compounds and the antioxidant capacity, with ethyl acetate extract identified as the most potent fraction. Additionally, it is the first to report cytotoxic and anti-inflammatory properties of CFSB in Bangladesh, highlighting its potential as a source of therapeutic bioactive compounds. Employing standardized biochemical tests, including DPPH (1,1-diphenyl-2-picrylhydrazyl), Folin-Ciocalteu, aluminum chloride, brine shrimp lethality, and protein denaturation, the research effectively assesses the antioxidant, antimicrobial, cytotoxic, and anti-inflammatory activities of the extracts, supporting their therapeutic and phytochemical significance.

Materials and Methods

Sources of chemicals

The compounds DPPH, gallic acid, quercetin, and ammonium molybdate tetrahydrate were procured from Sigma Chemicals (USA). Ascorbic acid and aluminum chloride were acquired from SD Fine Chem Ltd. (Biosar, India). Ethanol, Folin-Ciocalteu reagent, sodium carbonate, potassium acetate, and dimethyl sulfoxide (DMSO) were procured from Merck (Germany). All chemicals employed in the study were of analytical grade.

Collection and identification of the stem bark from the Cassia fistula plant

The stem barks of mature *C. fistula* plants were collected in May 2020 from the Jahangirnagar University campus, Bangladesh (GPS coordinates: https://maps.app.goo.gl/Xu1eNSWgtZudQvJRA?g_st=aw). The plant was authenticated by an expert taxonomist at the Bangladesh National Herbarium, Dhaka, Bangladesh, where voucher specimen No. 41562, was designated and the materials deposited for reference.

Crude extraction from Cassia fistula stem bark

The stem barks of *C. fistula* were sectioned into small portions and dried under ambient conditions in the shade. The dried material was thereafter pulverized to obtain a fine powder employing a mechanical grinder. Sequential extractions were performed at ambient temperature with n-hexane, chloroform ($CHCl_3$), ethyl acetate, and methanol (MeOH). Each solvent extract was condensed to increase its concentration and dried under reduced pressure using a rotary evaporator to remove residual solvents. The resulting extracts were labeled as follows: CF-1 (n-hexane extract), CF-2 ($CHCl_3$ extract), CF-3 (ethyl acetate extract), and CF-4 (MeOH extract). The schematic illustration of the crude extract preparation from the CFSB is presented in Figure 1.

Assessment of antioxidant activity by DPPH free radical scavenging assay

The antioxidant capacity of CFSB extracts was evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, as described by the standard method. DPPH is a persistent free radical characterized by a deep violet hue in solution owing to its absorption peak observed at 517 nm and serves as an electron acceptor. In the presence of antioxidants, they contribute electrons to DPPH, converting it into a colourless or light-yellow derivative, which reflects antioxidant-mediated free radical neutralization activity.[25,26] To perform the assay, a series of ascorbic acid dilutions at varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg/mL in methanol) and *C. fistula* extract solutions (1 mL) were independently prepared and added to 3 mL of 0.4 mM DPPH solution. The mixtures were incubated in the dark for 30 minutes, after which the absorbance was measured at 517 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu UV-1800, Kyoto, Japan), with ascorbic acid (AA) serving as the positive control. Every test was executed in three replicates. The inhibitory activity of DPPH was quantified employing the formula presented in Equation 1.[27]

$$\%\ inhibition = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100 \ldots\ldots\ldots\ldots\ldots Equation\ 1$$

The IC_{50} value, denoting the concentration required to neutralize 50% of DPPH radicals, was computed by linear regression of % inhibition versus concentration.[28] Each determination was carried out three times, and the average result was recorded.

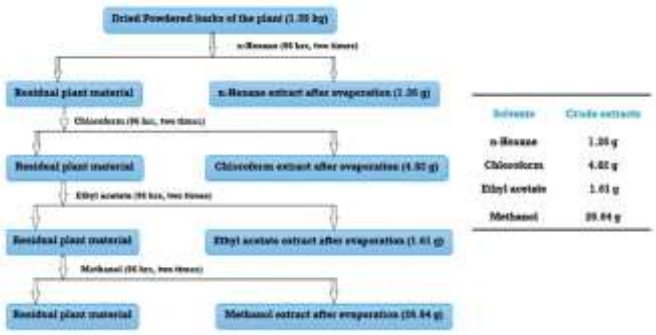

Figure 1: Schematic illustration of the preparation process for crude extracts from *Cassia fistula.*

Determination of total phenolic content

The total phenolic content (TPC) of the CFSB extracts was quantified using the Folin-Ciocalteu reagent (FCR) colourimetric assay. Folin-Ciocalteu reagent, a mixture of phosphomolybdate and phosphotungstate, reacts with phenolic and polyphenolic antioxidants to produce a colour change measurable by spectrophotometry.[29,30] Briefly, 1 mL of CFSB extract (200 µg/mL) or standard solution of gallic acid (prepared at concentrations of 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL) was dispensed into individual test tubes. Then, 5 mL of FCR and 7.5% Na_2CO_3 were added, followed by thorough mixing. The test tubes with standard solutions were incubated for 30 minutes, while those containing extract solutions were incubated for 1 hour at room temperature to allow the reaction to complete. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). A calibration curve was generated using gallic acid, and the TPC of the plant extracts was expressed in mg of gallic acid equivalents (GAE) per gram of plant extracts. The TPC in the plant extracts, expressed in GAE, was calculated employing Equation 2.

$$C = \frac{(c\ x\ V)}{m} \ldots\ldots\ldots\ldots\ldots\ldots Equation\ 2$$

Where C is the total phenolic content (mg GAE/g of extract), c is the concentration of gallic acid from the calibration curve (mg/mL), V is the volume of plant extract (mL), and m is the mass of the crude plant extract (g).[31]

Determination of total flavonoid content

The total flavonoid content (TFC) of the plant extracts was determined using a colourimetric method based on aluminum chloride complexation.[32] In this method, 1 mL of all plant extracts (200 µg/mL) and quercetin standard solution at varying concentrations (200, 100, 50,

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

25, 12.5, 6.25, and 3.125 µg/mL) was placed in different test tubes. To each tube, 3 mL of methanol, 200 µL of 10% aluminum chloride solution, and 200 µL of 1M potassium acetate solution were added, followed by the addition of 5.6 mL of distilled water. The reaction mixture was incubated for 30 minutes at room temperature, after which the absorbance was measured at 415 nm using a spectrophotometer, with a blank as the reference. Quercetin was used as the standard, and flavonoid content in the extracts was expressed as mg of quercetin equivalents (QE) per gram of extract. The TFC in the plant extracts, expressed in QE, was calculated using Equation 2.

$$C = \frac{(c\ x\ V)}{m} \ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 2$$

Where C is the total flavonoid content (mg QE/g of extract), c is the concentration of quercetin from the calibration curve (mg/mL), V is the volume of the extract (mL), and m is the mass of the crude plant extract (g).

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) of the extracts was determined using the phosphomolybdenum method as described by Prieto *et al*.[33] This assay measures the reduction of Mo (VI) to Mo (V) by antioxidants in the sample, resulting in the formation of a green phosphate/Mo(V) complex under acidic conditions. The complex exhibits maximal absorbance at 695 nm, indicating antioxidant activity. For the assay, 300 µL of each plant extract and ascorbic acid standard at various concentrations (200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL) was placed in individual test tubes. Then, 3 mL of phosphomolybdate reagent was added to each tube. The reaction mixtures were incubated at 95°C for 90 minutes to allow for a complete reaction. Absorbance was then measured at 695 nm using a spectrophotometer. The TAC of the extracts was expressed in ascorbic acid equivalents (AAE) and calculated using Equation 2.

$$C = \frac{(c\ x\ V)}{m} \ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 2$$

Where C represents the total antioxidant content (mg AAE/g of extract), c is the concentration of ascorbic acid from the calibration curve (mg/mL), V is the volume of the extract (mL), and m is the mass of the crude plant extract (g).

Assessment of cytotoxicity using the brine shrimp lethality bioassay

The cytotoxicity of CFSB extracts was assessed using the brine shrimp lethality test (BSLT). This bioassay is a rapid, cost-effective, and reliable method to screen bioactive compounds, utilizing the mortality of *Artemia salina* (brine shrimp) nauplii as a measure of cytotoxicity.[34] Figure 2 presents a schematic illustration of the BSLT employed to assess the cytotoxic activity of CFSB extracts. To prepare seawater, 38 g of non-iodized sea salt was dissolved in 1 L of distilled water. Brine shrimp eggs were added to one side of a tank filled with seawater and left to hatch under continuous aeration for 48 hours at room temperature. Nauplii were then attracted to light, separated from eggshells, and collected for the bioassay.

To prepare the sample, each plant extract (32 mg) was dissolved in 200 µl of DMSO and diluted to 20 mL with seawater, yielding a stock concentration of 1600 µg/mL. This solution was serially diluted to obtain concentrations ranging from 800 to 6.25 µg/mL. For each concentration, 5 mL of extract solution was mixed with 5 mL of seawater containing 10 nauplii. Vincristine sulfate served as a positive control, tested at concentrations of 10 to 0.157 µg/mL. A negative control containing 50 µl of DMSO in seawater with nauplii was used to ensure any observed mortality was solely due to the extract's cytotoxicity. The assay was performed by incubating the test tubes at room temperature, and after 24 hours, the surviving nauplii were counted using a magnifying glass. Mortality was calculated by utilizing Equation 3:

$$\%\ Mortality = \frac{No.\ of\ nauplii\ taken - No.of\ nauplii\ alive}{No.\ of\ nauplii\ alive} \times 100$$
……. Equation 3

The median lethal concentration (LC_{50}) was determined by linear regression, plotting % mortality against the log of extract concentrations.

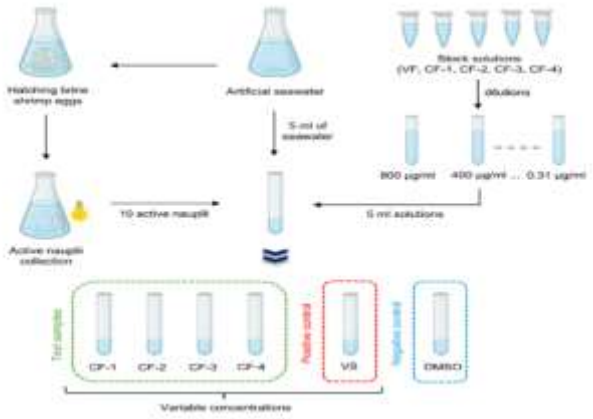

Figure 2: Schematic illustration of the brine shrimp lethality test (BSLT) for assessing the cytotoxicity of *Cassia fistula* stem bark.

Assessment of antimicrobial activity

The antimicrobial activity of the *C. fistula* extracts was evaluated using the disc diffusion method.[35] Four bacterial strains (*Bacillus megaterium*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*) and two fungal strains (*Aspergillus niger* and *Aspergillus flavus*) were tested. Sterile discs were loaded with 500 µg of each extract, while kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) served as positive controls for antibacterial and antifungal activities, respectively. The sample, control, and standard antibiotic discs were positioned on designated areas of agar plates that had been pre-inoculated with the test microorganisms. Plates were first refrigerated at 4°C for 24 hours to allow diffusion, then incubated at 37°C for another 24 hours. After incubation, antimicrobial activity was assessed by measuring the diameter of inhibition zones (mm) around each disc. The sensitivity of each microorganism to the extracts was compared with the inhibition zones produced by kanamycin and ketoconazole. All tests were performed in triplicate.

Assessment of the anti-inflammatory activity through the inhibition of bovine serum albumin denaturation

The anti-inflammatory activity of the extracts was evaluated by inhibiting protein denaturation, following standard protocols with minor modifications.[36] A 1 mL sample of each extract (aqueous and ethanolic) or diclofenac sodium (standard) at varying concentrations (100, 200, 500, and 1000 µg/mL) was mixed with 1 mL of 5% aqueous bovine serum albumin (BSA) solution. The mixture was incubated at 27°C for 15 minutes, with distilled water and BSA serving as the control. Protein denaturation was induced by heating the mixture at 70°C in a water bath for 10 minutes, then cooling it to room temperature. Absorbance was measured at 660 nm.

Assessment of anti-inflammatory activity through the inhibition of egg albumin denaturation

For egg albumin denaturation, the procedure was adapted from standard methods with slight modifications.[37] A 1 mL test solution of plant extract or diclofenac sodium (100-500 µg/mL) was combined with 1 mL of egg albumin solution (1 mM) and incubated at 27 ± 1°C for 15 minutes. The reaction mixture was then heated at 70°C for 10 minutes to induce denaturation, cooled to room temperature, and absorbance was recorded at 660 nm. Percentage inhibition of protein denaturation was calculated relative to the control, using the formula (Equation 4). All assays were conducted in triplicate, and the results were averaged.

$$\%\ inhibition = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100 \ldots\ldots\ldots Equation\ 4$$

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Results and Discussion

Antioxidant activity of the Cassia fistula stem bark extracts

Upon receiving an electron from an antioxidant compound (A-H), DPPH undergoes decolourization, changing from a deep purple to a pale-yellow DPPH-H (Figure 3A). This colour shift, indicative of free radical neutralization, can be quantitatively measured by changes in absorbance. DPPH, a stable nitrogen-centered free radical, transitions from violet to yellow upon reduction, either through hydrogen or electron donation. More decolourization indicates higher antioxidant activity, reflected by a lower IC_{50} value. Compounds capable of this reaction are considered effective antioxidants and radical scavengers. In Figure 3A, the percentage inhibition of all extracts and the standard, ascorbic acid, was plotted against concentration, from which IC_{50} values were calculated by linear regression shown in supplementary material (Figure S1) and presented in Figure 3B. The DPPH scavenging assay results showed that the IC_{50} values for the CF-3 and CF-4 were 8.45 µg/mL and 26.83 µg/mL, respectively, indicating strong antioxidant activity compared to the standard ascorbic acid (AA) (IC_{50} = 6.54 µg/mL). In contrast, CF-1 and CF-2 exhibited weaker scavenging activity, with IC_{50} values of 624.38 µg/mL and 351.57 µg/mL, respectively. This comparative study indicated that the CF-3 has the highest antioxidant activity among all extracts, while the CF-1 exhibited the lowest.

Total phenol content of the Cassia fistula stem bark extracts

The TPC of CFSB extracts was quantified employing the Folin-Ciocalteu reagent and expressed as gallic acid equivalents (GAE) per gram of extract. Total phenolic content values for all the extracts were calculated using the gallic acid standard curve ($y = 0.0075x + 0.0462$, $R^2 = 0.999$), and detailed calculations are provided in Supplementary Material (Table S2). Polyphenols' antioxidative properties stem from their high reactivity as hydrogen or electron donors, stabilizing and delocalizing free radicals (chain-breaking activity), and their capacity to chelate metal ions, which halts the Fenton reaction.[38] Figure 4A displays the calibration curve for gallic acid used to quantify TPC in each extract (CF-1, CF-2, CF-3, CF-4), and Figure 4B summarizes TPC values (mg GAE/g extract) for each sample. Phenolic compounds are known to counteract oxidation, a significant factor in atherogenesis, and studies link antioxidant activity with decreased risks of cardiovascular diseases, highlighting the importance of phenolic content.

The results of the present study indicated that the CF-3 exhibited the highest phenolic content at 175.53 ± 4.24 mg GAE/g, followed by the CF-4 at 127.53 ± 4.24 mg GAE/g. The higher TPC in these extracts suggests stronger antioxidant properties compared to the CF-1 and CF-2 extracts. Consequently, the antioxidant efficacy of CFSB extracts is as follows: ethyl acetate extract > methanol extract > chloroform extract > n-hexane extract. The prominent phenolic content in the ethyl acetate and methanol extracts implies significant antioxidant potential.

Total flavonoid content of the Cassia fistula stem bark extracts

The TFC of CFSB extracts was determined using the aluminum chloride colourimetric method. Flavonoid concentrations were quantified by constructing a standard curve with quercetin ($y = 0.008x + 0.0483$, $R^2 = 0.9989$) and expressed as quercetin equivalents (QE) per gram of extract. Detailed calculations for TFC in each extract are provided in Supplementary Material (Table S3). Known for their antioxidant properties, flavonoids are valuable indicators of the plant's potential antioxidative capacity, with prior studies showing a positive correlation between flavonoid content and antioxidant activity. This assay relies on forming a yellow complex between flavonoids and $AlCl_3$, which is measured spectrophotometrically to determine flavonoid presence.[39] Figure 5A displays the quercetin calibration curve (absorbance versus concentration) used for calculating TFC, and Figure 5B presents TFC values (mg QE/g extract) for each sample.

The results indicate that the flavonoid content across extracts varies significantly, with the CF-3 showing the highest TFC at 319.19 ± 2.65 mg QE/g, followed by CF-1 (39.50 ± 7.51 mg QE/g), CF-2 (23.25 ± 1.33 mg QE/g), and CF-4 (4.50 ± 0.44 mg QE/g) extracts. The high flavonoid content in the CF-3 suggests a robust antioxidant capacity compared to the other extracts, reinforcing its potential as a superior antioxidant property among CFSB extracts.

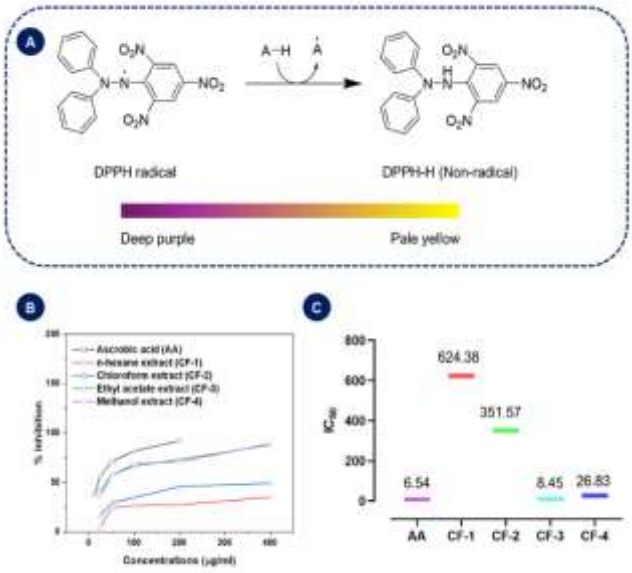

Figure 3: *Antioxidant activity of the Cassia fistula stem bark extracts through the* DPPH free radical scavenging assay. A: Schematic representation of the reaction mechanism; B: Plot of % inhibition versus concentration for ascorbic acid (AA) and plant extracts (CF-1, CF-2, CF-3, CF-4); C: Comparative IC_{50} values for AA and the extracts (CF-1, CF-2, CF-3, CF-4), indicating antioxidant potency; DPPH: 1,1-diphenyl-2-picrylhydrazyl; CF-1: n-hexane extract; CF-2: $CHCl_3$ extract; CF-3: ethyl acetate extract; CF-4: MeOH extract.

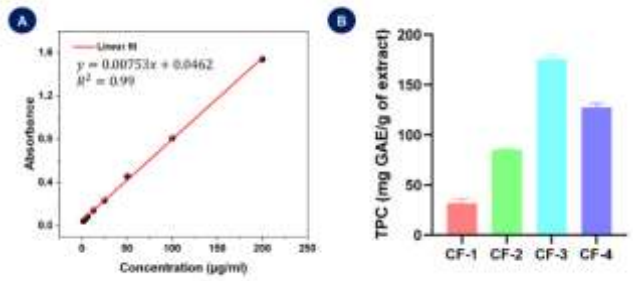

Figure 4: Total phenolic content (TPC) of *Cassia fistula* stem bark extracts.
A: Calibration curve of gallic acid showing absorbance versus concentration; B: TPC of the extracts (CF-1, CF-2, CF-3, CF-4) expressed as mg GAE/g extract; CF-1: n-hexane extract; CF-2: $CHCl_3$ extract; CF-3: ethyl acetate extract; CF-4: MeOH extract.

Total antioxidant capacity of the Cassia fistula stem bark extracts

The antioxidant activity of phenolic compounds is largely attributed to their redox properties, which enable them to absorb and neutralize free radicals, quench singlet and triplet oxygen, and decompose peroxides. In this study, the TAC of CFSB extracts was assessed using the phospho-molybdenum method, which involves the reduction of Mo

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

(VI) to Mo (V) by antioxidant compounds, forming a green phosphate/Mo (V) complex with maximal absorption at 695 nm. Total antioxidant capacity values were expressed as ascorbic acid equivalents (AAE) per gram of extract, calculated for all extracts from the ascorbic acid standard curve ($y = 0.0021x + 0.0345$, $R^2 = 0.9841$). Detailed calculations are provided in Supplementary Material (Table S4). While phenolics are significant contributors to antioxidant activity, other secondary metabolites, including volatile oils, carotenoids, and vitamins, may also contribute to the antioxidant properties of plant extracts. The redox characteristics of phenolic compounds enable them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

Figure 6A shows the ascorbic acid calibration curve, used for calculating TAC, and Figure 6B presents the TAC values (mg AAE/g extract) for each extract (CF-1, CF-2, CF-3, and CF-4). The TAC of CFSB extracts was observed to be 60.71 ± 3.37 mg AAE/g for CF-1, 204.76 ± 5.05 mg AAE/g for CF-2, 415.48 ± 3.37 mg AAE/g for CF-3, and 548.81 ± 6.73 mg AAE/g for CF-4. These results demonstrate that the CF-4 exhibits the highest total antioxidant capacity, followed closely by the CF-3, both of which show significantly greater antioxidant potential than the CF-1 and CF-2.

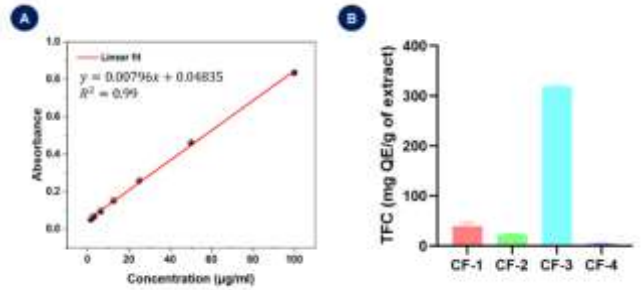

Figure 5: Total flavonoid content (TFC) of *Cassia fistula* stem bark extracts.
A: Calibration curve of quercetin, illustrating absorbance versus concentration; B: TFC (expressed as mg QE/g extract) for samples CF-1, CF-2, CF-3, and CF-4; CF-1: n-hexane extract; CF-2: $CHCl_3$ extract; CF-3: ethyl acetate extract; CF-4: MeOH extract.

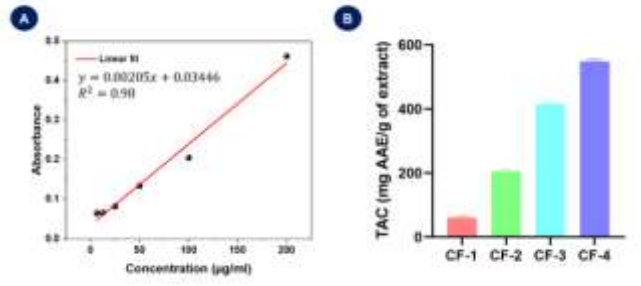

Figure 6: Total antioxidant capacity (TAC) of *Cassia fistula* stem bark extracts.
A: Calibration curve of ascorbic acid, illustrating absorbance versus concentration; B: TAC (expressed as mg AAE/g extract) for samples CF-1, CF-2, CF-3, and CF-4; CF-1: n-hexane extract; CF-2: $CHCl_3$ extract; CF-3: ethyl acetate extract; CF-4: MeOH extract.

Cytotoxic activity of the Cassia fistula stem bark extracts

The BSLT is a versatile method commonly used to assess bioactivity in crude extracts, providing a reliable indicator of cytotoxic and pesticidal potential.[40] In this study, the cytotoxic activity of each extract was evaluated using the BSLT method. Linear regression analysis of % mortality vs. concentrations for the standard vincristine sulfate (VA) and all extracts (CF-1, CF-2, CF-3, and CF-4) is presented in Supplementary Material (Figure S2). Figure 7A illustrates the relationship of % mortality versus concentration for both vincristine sulfate and each CFSB, while Figure 7B shows the LC_{50} values for VA and the extracts for comparative analysis.

The cytotoxicity results indicate that the CF-1 exhibited moderate cytotoxicity with an LC_{50} value of 30.70 µg/mL, compared to the standard vincristine sulfate (VS), which showed a much lower LC_{50} value of 3.501 µg/mL. The other extracts displayed non-significant cytotoxicity relative to both the standard and the CF-1. These findings suggest that while the CF-1 possesses moderate cytotoxic potential, the other extracts lack substantial cytotoxic effects.

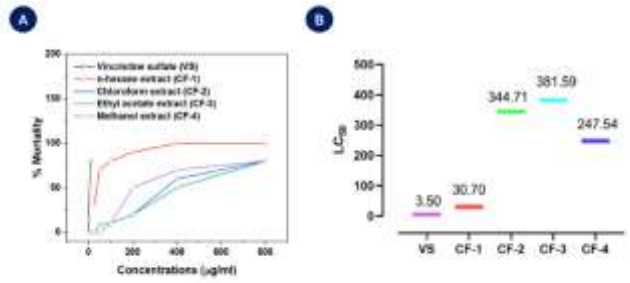

Figure 7: *Cytotoxic potential of the Cassia fistula stem bark extracts.*
A: % mortality versus concentration for standard vincristine sulfate (VA) and *Cassia fistula* stem bark extracts (CF-1, CF-2, CF-3, CF-4); B: Comparative LC_{50} values for vincristine sulfate and each extract.

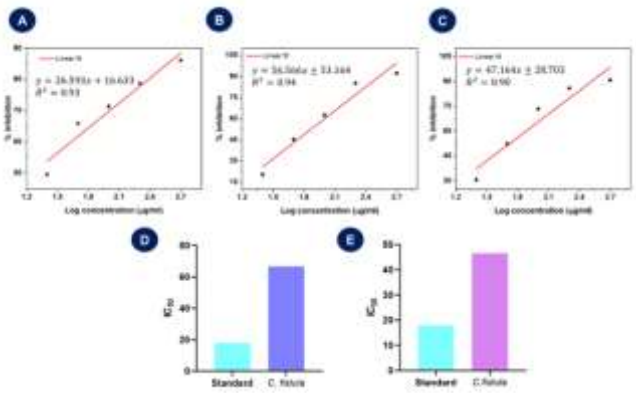

Figure 8: Calibration curves showing % inhibition versus concentration.
A: standard diclofenac sodium; B: methanol extract of *Cassia fistula* stem bark in the inhibition of BSA denaturation; C: methanol extract of *Cassia fistula* stem bark in the inhibition of EA denaturation; Plots comparing IC_{50} values between the standard and methanol extract of *Cassia fistula* stem bark for the inhibition of D: BSA (bovine serum albumin) denaturation and; E: EA (egg albumin) denaturation.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Antimicrobial activity of the Cassia fistula stem bark extracts

The antimicrobial activity of CFSB extracts was evaluated using the disc diffusion method. Four pathogenic bacterial strains and two fungal strains were tested for sensitivity to the extracts (500 µg/disc), with inhibitory zones measured in millimeters. Kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) served as standards for antibacterial and antifungal screening, respectively. Each experiment was performed in triplicate, and the results are summarized in Table 1. In the antibacterial assay, the CF-2 and CF-3 extracts exhibited mild to moderate inhibitory activity against the Gram-positive bacteria, *Bacillus megaterium* and *Staphylococcus aureus*, compared to kanamycin. Additionally, the CF-3 showed mild to moderate activity against the Gram-negative bacteria, *Salmonella typhi,* and *Escherichia coli*, whereas the CF-2 showed no activity against these strains. The CF-1 exhibited limited activity against the bacterial strains tested, while the CF-4 showed no antibacterial activity. For antifungal screening, none of the extracts demonstrated activity against *Aspergillus flavus*. However, the CF-2 and CF-3 extracts exhibited mild to moderate inhibitory effects against *Aspergillus niger*, relative to ketoconazole. These findings suggest that the CF-3 of CFSB may serve as a promising source of antimicrobial compounds, particularly against specific bacterial and fungal pathogens.

Table 1: Antimicrobial screening of extracts from the stem barks of *Cassia fistula*

Sample (500 µg/disc)		Diameter of Zone of Inhibition (mm)					
		Bacterial Strain				Fungal Strain	
		Gram Positive		Gram Negative			
		B. megaterium	*S. aureus*	*S. typhi*	*E. coli*	*A. niger*	*A. flavus*
n-Hexane extract		8	8	ns	6	ns	ns
Chloroform extract		13	10	ns	ns	11	ns
Ethyl acetate extract		10	11	12	10	9	ns
Methanol extract		ns	ns	ns	ns	ns	ns
Positive Control (30 µg/disc)	KM	34	23	25	32	nd	nd
	KC	nd	nd	nd	nd	24	22

KM: kanamycin; KC: ketoconazole; ns: no sensitivity; nd: not done.

Anti-Inflammatory activity of the Cassia fistula stem bark extracts

Protein denaturation, particularly under heat-induced conditions, is a hallmark process in inflammation, often leading to cellular damage and dysfunction.[41] In this study, the ability of test samples to inhibit the denaturation of two proteins, bovine serum albumin (BSA) and egg albumin (EA) was evaluated as a measure of their anti-inflammatory potential. The anti-denaturation effects of these samples were quantified using IC_{50} values, with lower IC_{50} values indicating greater protein stabilization. The test samples included a methanol extract of CFSB, evaluated in comparison to the reference anti-inflammatory drug, diclofenac sodium.

Figures 8A-C show the calibration curve plots of % inhibition versus concentration for standard diclofenac sodium and the methanol extract of CFSB in the inhibition of BSA and EA denaturation, respectively. From these calibration plots, IC_{50} values were calculated for both the standard and *C. fistula* methanol extract, as displayed in Figures 8D and 8E. The results showed a significant decrease in IC_{50} values for the *C. fistula* methanol extract relative to the control, indicating effective inhibition of heat-induced protein denaturation. This finding suggests that the methanol extract exhibits moderately significant anti-inflammatory activity, approaching the effectiveness of diclofenac sodium. These results highlight the potential of CFSB as a natural anti-inflammatory agent, warranting further investigation into its bioactive compounds and mechanisms of action.

Conclusion

This study comprehensively demonstrated the bioactive potential of CFSB through systematic evaluation of its antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities. The ethyl acetate extract exhibited the highest phenolic and flavonoid content, correlating strongly with its antioxidant potency. Moderate cytotoxic and anti-inflammatory effects were observed in the n-hexane and methanol extracts, respectively, indicating diverse pharmacological attributes across solvent fractions. The antimicrobial assays revealed selective inhibition of bacterial and fungal pathogens, supporting the plant's ethnomedicinal relevance. Collectively, these findings highlight CFSB as a promising natural source of bioactive compounds with therapeutic potential, meriting further phytochemical characterization and mechanistic studies for possible drug development applications.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors are grateful to the Research Chemistry, Jahangirnagar University, Savar, Dhaka, and the Department of Pharmacy, Gono Bishwbidyalay, Savar, Dhaka, Bangladesh, for providing laboratory facilities to conduct the research.

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