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Exploring Bioactive Compounds in *Cajanus cajan* (L.) Millsp. Stem and their biological activities: Integration of GC-MS and LC-MS Techniques

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ABSTRACT

Cajanus cajan, a well-known legume crop with nutritional significance, has been used traditionally for various therapeutic purposes. Despite its ethnomedicinal importance and rich metabolite composition, there have been limited investigations into the secondary metabolites present in its stems using advanced analytical techniques. This study aimed to enhance our understanding of the chemical composition and biological activities of underutilized *C. cajan* stems. GC-MS & LC-MS analysis revealed flavonoids as the stem's predominant secondary metabolites. The methanol extract exhibited potent antibacterial activity against *E. coli*, *S. aureus*, and *C. albicans*. Major bioactive compounds, including rutin, apiin, quercetrin, and aesculine, contributed to these beneficial effects. The study also revealed a high total phenolic content of 121.5 mg GAE/g in the stem extract, further establishing *C. cajan* stems as a valuable source of therapeutic molecules.

Keywords: *Cajanus cajan*, GC-MS, LC-MS, Antioxidant, Antimicrobial, Total phenolic content.

INTRODUCTION

The presence of significant secondary metabolites has primarily been studied in plants with economic, nutritional, and therapeutic importance. Phytocompounds and secondary metabolites have played a pivotal role in the discovery of therapeutically valuable molecules throughout history. Medicinal plants serve as a rich source of natural products containing biologically active compounds, many of which have laid the foundation for novel pharmaceuticals [1]. Given the increasing prevalence of life-threatening diseases, understanding the phytochemical composition and bioactivities of nutritional and medicinal plants has become crucial in addressing current health challenges [2]. *Cajanus cajan*, also known as pigeon pea or red gram, is a perennial legume plant belonging to the Fabaceae family. Originally from India and widely cultivated in tropical and subtropical regions, *C. cajan* holds significant agricultural importance as a staple food crop. Its edible seeds, leaves, and young pods have been utilized for centuries, contributing to its reputation as a protein-rich crop mainly grown in semi-arid region. On a global scale, it stands as the sixth most cultivated pulse, with particular prominence in Asia, it holds the third position among the most cultivated crops [3]. The study and utilization of such plants and their phytochemical properties have the potential to offer valuable solutions in the fields of medicine and nutrition. *C. cajan*, a versatile plant with a woody stem, can grow 1 to 4 meters tall. It features compound leaves with three leaflets and produces yellow or orange flowers in clustered arrangements. The plant yields elongated, curved pods containing pea-like seeds. Deep-rooted and nitrogen-fixing, it thrives up to 2 meters in the soil. This leguminous plant is renowned for its nutritional value, abundant in proteins, crude fiber, iron, sulfur, calcium, potassium, manganese, and water-soluble vitamins. Its wide variety of secondary metabolites has piqued interest in exploring its potential medical applications, making it a valuable focus of study [4]. Literature surveys reveal that while the leaves and seeds of plants are extensively studied for their phytochemical content, the potential of stems is often overlooked or underutilized. *C. cajan* stems, commonly used for construction and fuel, also possess medicinal uses. Decoctions from the leaves and stems are employed to alleviate various conditions like sore throat, cough, intestinal worms, skin irritations, and ulcers [5]. Across different ethnic groups and localities, traditional remedies using the stems and leaves include treatments for malaria, dizziness, measles, and eye infections. Pharmacological studies have shown significant medicinal properties in *C. cajan*, with reports of antidiabetic, antioxidant, and antimicrobial activities in various parts, such as seeds for menstrual problems, gastrointestinal disorders, dizziness, and diabetes, and leaves for malaria, ulcers, and measles. Additionally, the stems are traditionally used for toothache, wound healing, and stomatitis treatment [6]. Numerous studies have explored the pharmacological properties and chemical composition of *C. cajan*, focusing on its seeds, leaves, and roots.

However, there remains a research gap regarding the stem, with limited information on its chemical and biological characteristics. This study aimed to fill that void by investigating the chemical profile and biological properties of the stem. Previous research revealed the presence of various compounds, such as terpenoids, alkaloids, flavonoids, anthraquinones, phenols, saponins, and tannins, through phytochemical screening. In our earlier study, we used GC-MS and LC-MS techniques to conduct a phytochemical investigation of *C. cajan* leaves [7]. This research work is part of our continuous endeavor to explore untapped phytochemical and their respective biological activities from underutilized parts of legumes of Fabaceae family [8-10]. This article serves as an expansion of earlier investigations, utilizing GC-MS and LC-MS techniques on a methanolic extract of *C. cajan* stem, while also studying its antioxidant and antibacterial properties.

MATERIAL AND METHODS

Chemicals and Reagents

All chemicals and reagents employed in this research were of analytical grade, sourced from Merck. Methanol utilized for extraction was of HPLC grade.

Plant Material

The whole plant of *Cajanus cajan* (L.) Millsp, was collected at a location within Sillod. (Latitude N 20° 19', Longitude E 75° 39') District: Aurangabad, India January and February, 2018. The sample was separated into leaves, stems, and seeds by hand. The stems of plant were harvested. The plants were authenticated by the Dr. J. S. Ambhore Professor Department of Botany, Indraraj College, Sillod, where voucher specimens were deposited for future reference. The stems were air-dried in shade until they reached a constant weight, then finely ground into a powder using an electric blender to enhance surface area for extraction. The labeled powdered plant material was then stored in a sealed, dry container at room temperature until needed for subsequent procedures.

Preparation of *Cajanus cajan* stem extract

40 grams of dried stem powder was mixed with 200 mL of methanol and subjected to hot continuous extraction via Soxhlet apparatus at 60°C for 6-8 hours. The resulting extracts were then concentrated under reduced pressure using a rotary evaporator. The concentrated extracts were stored in airtight containers at 4°C until ready for GC-MS and LC-MS analysis.

Preliminary phytochemical screening of the extract

Following the extraction process, the obtained extract underwent preliminary phytochemical screening according to standard procedures. Various phytochemical screening tests were conducted to determine the chemical composition of the extract.

GC-MS experimental system and measurements

The Thermo Scientific TSQ-800 GS-MS instrument was used for GC-MS analysis, which was connected with a silica capillary column TG-5-MS (dimensions-30 m × 0.25 mm, film thickness 0.25 µm). The electron impact ionization system was set at an ionizing energy of 70 eV with a scanning mass range of 50-700 (m/z), and helium carrier gas was used at a flow rate of 1 mL/minute. The phytochemical analysis procedure began with the oven temperature being initiated at 60°C for 2 min, followed by an increase up to 280°C at a rate of 5°C/min, and then maintained isothermally for 10 min. The injector port, ion source, and detector temperatures were set at 250°C, 260°C, and 280°C respectively. The GC run time was 21 minutes. The names, molecular weights, and structures of the components were retrieved from the database of the National Institute of Standards & Technology (NIST) Library.

LC-MS experimental system and measurements

LC-ESI-Q-TOF MS (Agilent Technologies 6550 i-funnel) system at SAIF, IIT, Bombay was used to analyze the methanol extract of the sample for the recognition of active phytoconstituents. The two-solvent elution approach included a gradient system of 0.1 % formic acid in water (A) and 90 % acetonitrile + 10 % water + 0.1 % formic acid (B) at a flow rate of 0.3 mL/min and an injection volume of 5 µL. The elution was carried out in a step gradient manner as follows: 95:5 for 25 minutes, 5:95 for 5 minutes, and finally back to initial composition 95 % of A and 5 % of B in 1 minute, which was maintained at the same composition for 5 minutes. The compounds were ionized using the dual positive (+ve) and negative (-ve) modes of ESI and channelized using i-funnel Q-TOF. The Q-TOF MS source parameters were set as follows: capillary voltage 3500 V, mass range 120-1000 m/z, gas flow 13 L/min, gas temperature 250°C, sheath gas temperature 250°C, sheath gas flow 11, nebulizing gas pressure 35 (psig), skimmer 65 V, fragment 175 V, RF peak 750 V. The Mass Hunter software was used for profiling, characterizing, identifying, and quantifying the compounds present in the extract via high-definition MS and MS/MS. The MS spectra received from the analyzed samples were explored against the Metlin database to find the presumptive compounds present in the sample.

Evaluation of antibacterial and antifungal activity

For the antimicrobial study, the Microbroth dilution method was utilized to ascertain the minimum inhibitory concentration (MIC) of the methanolic extract derived from *Cajanus cajan* stems. Seven pathogens were employed to assess the antimicrobial efficacy of the plant extract, with all MTCC strains obtained from the Institute of Microbial Technology in Chandigarh. The antibacterial activity of the extract was evaluated against four bacterial strains, including two Gram-negative bacteria (*Escherichia coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 444) and two Gram-positive bacteria (*Staphylococcus aureus* MTCC 96 and *Streptococcus pyogenes* MTCC 442). Additionally, five standard drugs—Gentamycin, Ampicillin, Chloramphenicol, Ciprofloxacin, and Norfloxacin—were studied for comparison purposes. The antifungal activity of the plant extract was analyzed against three fungal strains: *Candida albicans* MTCC227, *Aspergillus Niger* MTCC282, and *Aspergillus clavatus* MTCC1323, with comparison made using two standard drugs, nystatin and griseofulvin. The experimental protocol included the use of growth media, sample preparation, and MIC determination following standard procedures. MICs were determined through serial dilution of the plant extract ranging from 6.250 µg/mL to 1000 µg/mL, with Muller Hinton Broth and DMSO serving as nutrient medium diluents, respectively. MIC values were determined based on the absence of visible growth of the tested organisms upon microscopic evaluation of the culture tubes and expressed in µg/mL. Each assay was conducted in triplicate, and data were presented as mean values.

Total Phenolic content Analysis

The spectrophotometric method with slight modification [12] was used to determine the total phenolic content assay using Folin-Ciocalteu reagent. The total phenolic content was quantified by mixing 1 mL of aliquots of concentration range from 50 to 500 µg/mL Gallic acid solution with 5.0 mL of Folin Ciocalteu reagent (diluted tenfold). After 5 minutes, 5 mL of 20% (w/v) Na_2CO_3 solution was added to the mixture, and the absorbance was measured after 30 min at 765 nm. The total phenolic content was calculated separately for methanolic extracts (1 gm/100 ml or 2 mg/1mL) using the same reagents as used for constructing the calibration curve. After 1 hour, the absorbance was measured to determine the total phenolic contents in both extracts separately using the formula TPC = C1 X V/M where TPC is the total phenolic content in mg/g in GAE (Gallic acid equivalent), C1 is the concentration of Gallic acid established from the calibration curve in mg/ml, V is the volume of extract in ml, and m is the weight of the plant extract in g.

Preliminary Phytochemical Screening

Identifying bioactive compounds aids understanding traditional folk medicine and uncovers new therapeutic possibilities. Preliminary phytochemical test was found to be positive for flavonoids, coumarins, alkaloids and glycosides.

Total Phenolic content Analysis

Phenolic compounds, abundant in plants, exhibit diverse biological activities and are promising for new drug development. *C. cajan* stem analysis revealed high phenolic content 121.5 mg GAE/g, (Figure 1) linked to health benefits like antioxidants and anti-inflammatories, surpassing previous findings [13].

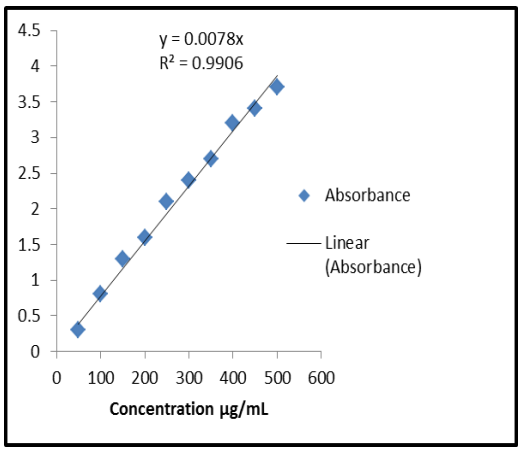

Figure 1: Gallic acid standard curve

GC-MS Analysis

Four compounds of different chemical families were identified in the methanolic extract using GC-MS, including heptacosane, hydroperoxide, 1, 4-dioxan-2-yl, hexadeconoic acid, 2,3-dihydroxypropyl ester and 1-iodo-2-methylundecane. GC-MS chromatogram of extract depicted in Figure 2 and identified compounds are tabulated in Table 1.

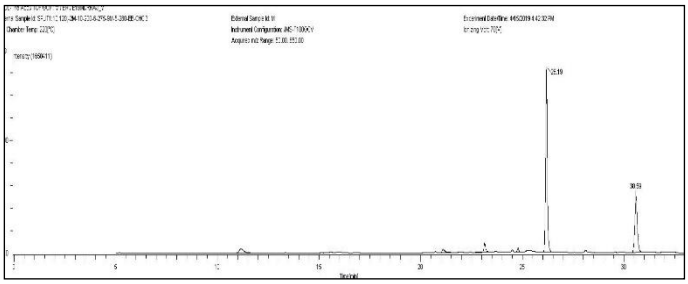

Figure 2: *C. cajan* stem methanolic extract Gas Chromatography chromatogram

Table 1: GC-MS analysis of *C. cajan* stem methanolic extract.

S. No	Probable Compound	R.T. (Min)	Molecular mass (g/mole)	Molecular Formula	Compound Nature
1	Heptacosane	11.16	380	$C_{27}H_{56}$	Volatile oil
2	Hydroperoxide, 1,4-dioxan-2-yl	21.10	120	$C_4H_8O_4$	--
3	Hexadeconoic acid, 2,3-dihydroxypropyl ester	22.92	330	$C_{19}H_{38}O_4$	Fatty acid ester
4	1-iodo-2-methylundecane	23.14	296	$C_{12}H_{25}I$	Iodoalkane

LC-MS Analysis

LC-MS analysis of *C. cajan* stem's methanol extract identified 20 compounds, including flavonoids, phenolics, glycosides, terpenoids, amines, and more in positive and negative mode of ionization . (Figure 3 a and 3 b) (Table 2) Flavonoids were prominent, suggesting potential antioxidant activity and therapeutic benefits.

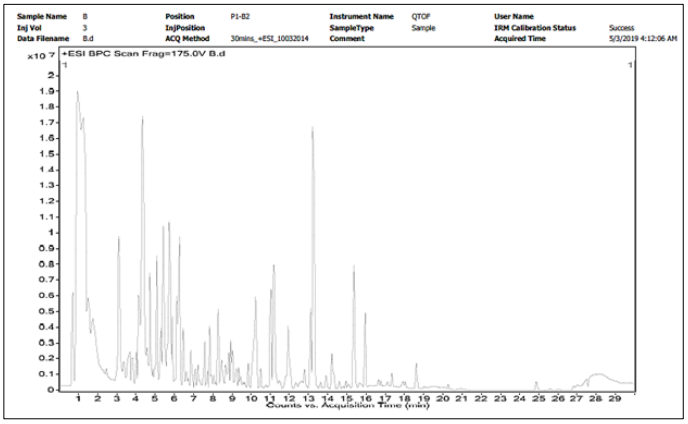

Figure 3 (a): *C. cajan* stem methanolic extract LC-MS chromatogram acquired in a positive mode of ionization

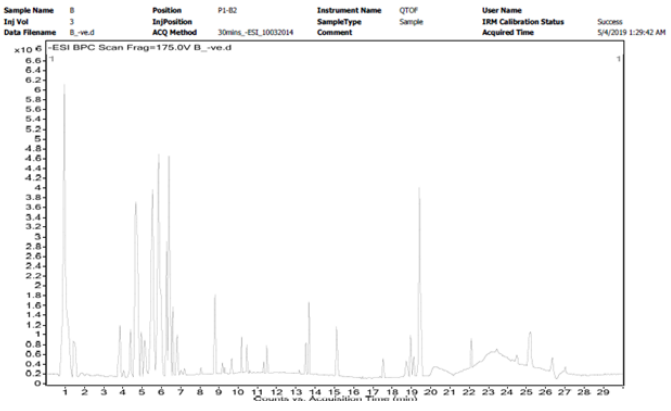

Figure 3 (b): *C. cajan* stem methanolic extract LC-MS chromatogram acquired in a negative mode of ionization

Table 2: Secondary metabolites from *C. cajan* stem by LC-MS analysis.

S. No.	Compound	Retention time	Molecular mass	Molecular Formula	m/z	Chemical Nature	Ionization
1	Hexanoylglycine	1.56	173.21	$C_8H_{15}NO_3$	156.10		
2	Tyramine	3.11	137	$C_8H_{11}NO$	120.08	Amine	$[M-H]^+$
3	16-Hydroxy-4-carboxyretinoic acid	4.40	344	$C_{20}H_{24}O_5$	349.13	Isoprenoid	$[M-H]^+$
4	Arbutin	4.70	272	$C_{12}H_{16}O_7$	295.07	Glycoside	$[M-H]^+$
5	Aesculin	4.73	340	$C_{15}H_{16}O_9$	323.07	Coumarin	$[M-H]^+$
6	Podophyllotoxin	4.91	414	$C_{22}H_{22}O_8$	397.12	----	$[M-H]^+$
7	Rutin	4.96	610	$C_{27}H_{30}O_{16}$	611.16	Flavonoid	$[M-H]^+$
8	Apiin	5.81	564	$C_{26}H_{28}O_{14}$	565.14	Flavonoid	$[M-H]^+$
9	Quercetrin	5.95	448	$C_{21}H_{20}O_{11}$	449.10	Flavonoid	$[M-H]^+$
10	Cosmosiin	6.77	432	$C_{21}H_{20}O_{10}$	433.11	Flavone	$[M-H]^+$
11	Dihydrokaempferol	6.88	288	$C_{15}H_{12}O_6$	271.06	Flavanone	$[M-H]^+$
12	Lecanoric acid	8.16	318	$C_{16}H_{14}O_7$	318.07		$[M-H]^+$
13	Koparin 2'-Methyl Ether	8.36	314	$C_{17}H_{14}O_6$	315.08	Flavone	$[M-H]^+$
14	7,2'-Dimethoxyflavone	8.43	282	$C_{14}H_{14}O_4$	283.09	Flavone	$[M+H]^-$
15	2-Ethoxycarbonyl-5,7-Dihydroxy-8,3',4',5'-Tetramethoxyisoflavone	9.00	446	$C_{22}H_{22}O_{10}$	447.12	Flavone	$[M+H]^-$
16	Epigallocatechin	9.01	306	$C_{15}H_{14}O_7$	329.06		$[M+H]^-$
17	Tamarixetin	9.42	316	$C_{16}H_{12}O_7$	317.16	Flavone	$[M+H]^-$
18	Lecanoric acid	10.31	318	$C_{16}H_{14}O_7$	301.07	Polyphenol	$[M+H]^-$
19	7-hydroxy-2'-methoxy-isoflavone	11.49	268	$C_{16}H_{12}O_4$	269.08	Flavone	
20	Ribonic acid	1.01	166	$C_5H_{10}O_6$	165.04		[M+H]

Antibacterial activity

Anti-bacterial activity of stem extract was evaluated against gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*). *C. cajan* stem methanolic extract (CCSME) demonstrated significant antibacterial activity, particularly against *E. coli* and *S. aureus*, (with a minimum inhibitory concentration (MIC) of 50 µg/ml) surpassing ampicillin and comparable to chloramphenicol. Figure 4 illustrates the extract's antimicrobial potential, highlighting its minimum inhibitory and bactericidal concentrations. Additionally, CCSME displayed potent antifungal effects against *C. albicans*, with an MFC of 200 µg/ml, outperforming griseofulvin. (Figure 5)

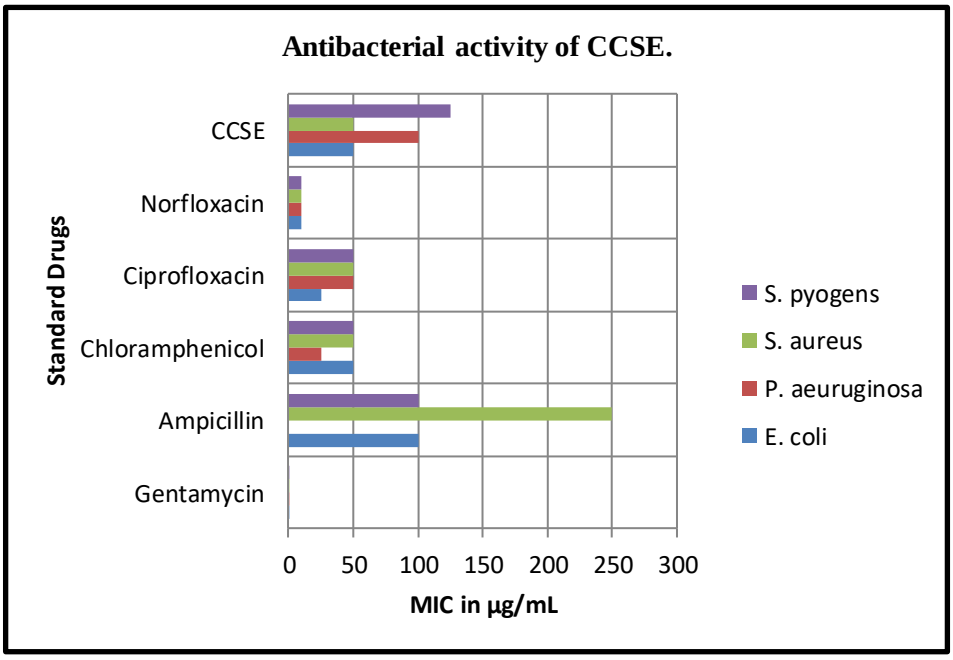

Figure 4: Antibacterial activity of *C. cajan* stem methanolic extract against selected bacterial strains

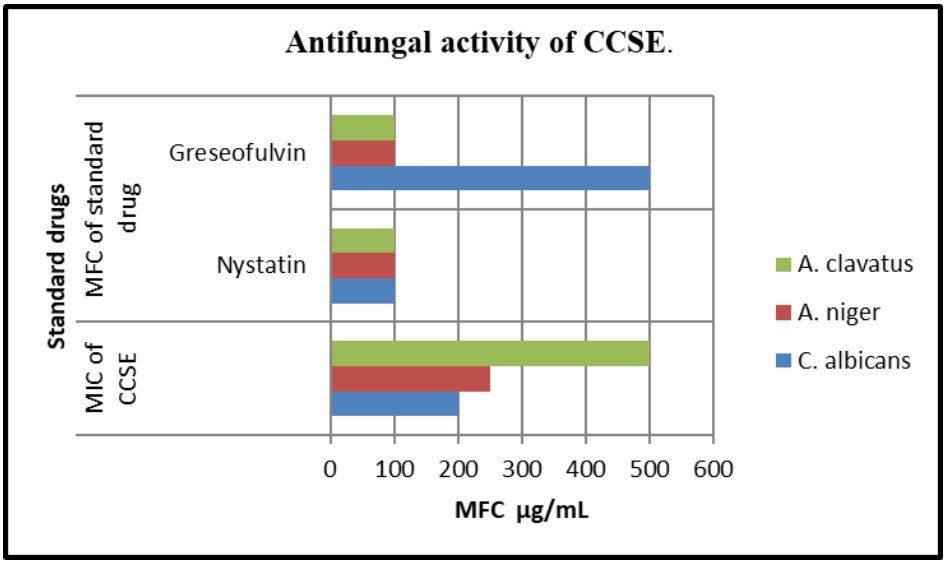

Figure 5: Antifungal activity of *C. cajan* stem methanolic extract against selected fungal strains

DISCUSSION

The preliminary phytochemical analysis revealed the presence of medicinally important secondary metabolites. Which were confirmed by hyphenated chromatographic techniques like GC-MS and LC-MS. The analysis of *C. cajan* stem extract using GC-MS and LC-MS techniques provided valuable insights into its phytochemical composition. LC-MS was effective in detecting polar and non-volatile compounds, while GC-MS identified volatile and semi-volatile constituents. GC-MS analysis identified heptacosane, which has been previously reported in another plant's alcoholic extract [14] and suggested as a tool to combat multidrug resistance in acute myeloid leukemia [15]. Hexadecanoic acid, 2,3-dihydroxypropyl ester, a fatty acid ester with antibacterial and anticancer properties, was also identified [16]. Previous studies on the GC-MS analysis of *C. cajan* stem reported other constituents like N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-2-amino acetamide, cyclopropane butanoic acid, 2-(4-nitrophenyl)-2-oxoethyl 3-phenyl propanoate, and heptanoic acid [17]. LC-MS analysis of the extract demonstrated prominent presence of flavonoids. Notably, rutin and quercitrin showed analgesic, antiarthritic, antidiabetic, and anticancer properties [18]. Arbutin, recognized for skin-whitening, [19] and aesculin, known for anti-inflammatory and antioxidant effects, were also found. The presence of secologanin, a monoterpenoid compound, was also detected. These findings shed light on the diverse secondary metabolites in the *C. cajan* stem, providing insights into its potential bioactivities and medicinal applications. Plant-derived compounds have emerged as promising antimicrobial agents to combat pathogenic microorganisms. *C. cajan* stem methanolic extract exhibited significant antibacterial activity, particularly against *E. coli* and *S. aureus*. Moreover, extract displayed potent antifungal effects against *C. albicans*. In overall extract demonstrated better antimicrobial activity compare to some standard drugs used. LC-MS analysis identified flavonoids as the extract's rich source, known for their strong antimicrobial properties [20]. These findings underscore the potential of stem extract as a well-tolerated and effective natural antimicrobial agent against harmful pathogens, opening avenues for future therapeutic applications.

CONCLUSION

This study represents the first application of a combined GC-MS and LC-MS approach to analyze the chemical composition of *C. cajan* stem extract. The extract demonstrated a substantial presence of flavonoid and phenolic compounds, along with remarkable antimicrobial activities. Identifying the specific flavonoids responsible for these biological effects is essential. The results support the traditional medicinal use of *C. cajan* stems and indicate their potential as a valuable source of natural antimicrobial agents. These findings lay the groundwork for further research and potential therapeutic applications of *C. cajan* stem compounds.

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Conflict of interest

The authors declared no conflict of interest.

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