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GC-MS Chemical Profiling and *In vitro* Biological Activities of Leaf Extracts of *Cymbidium aloifolium* (L.) Sw. (Orchidaceae)

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ABSTRACT

In present study, various solvent extracts of *Cymbidium aloifolium* were analyzed for their phytochemical profiles, and antimicrobial and antioxidant activities. The antimicrobial activity was tested using the agar well diffusion method against four pathogens: *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Streptococcus mutans*. The antioxidant activity was evaluated using the DPPH scavenging assay. Additionally, GCMS analysis was conducted to determine the chemical composition of the extracts.The methanolic extract demonstrated the highest antioxidant activity, with an IC_{50} value of 20.45±1.02 µg/ml, whereas the acetone extract showed the lowest antioxidant activity, with an IC_{50} value of 74.82±1.64 µg/ml. Methanol extracts also exhibited strong antibacterial activities, with inhibition zones ranging from 6.3±0.57 to 18.3±0.57 mm, while the aqueous extracts showed no antimicrobial activity. GCMS analysis of the methanolic extract of *C. aloifolium* identified nine components, with 1,3-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester (49.19%) and γ-Sitosterol (19.89%) being the most prominent. These findings suggest that the methanol extract of *C. aloifolium* leaves is a rich source of bioactive compounds with notable antioxidant and antimicrobial properties, making it a potential source for the development of treatments for various diseases.

Key words: Phytochemicals, DPPH assay, GCMS analysis, antioxidant activities

INTRODUCTION

Natural products derived from higher plants serve as a promising source of antimicrobial agents with unique mechanisms of action. These compounds are effective in treating infectious diseases and often reduce the side effects associated with synthetic antimicrobials. Traditional medicinal systems, such as Ayurveda, have long promoted the use of plant extracts to address various ailments. Plants produce secondary metabolites like alkaloids, phenolics, flavonoids, saponins, tannins and glycosides to protect themselves from predators and environmental stressors, which also play a crucial role in herbal medicine. These phytochemicals exhibit diverse pharmacological properties, such as antimicrobial, antifungal, anti-diabetic, anti-inflammatory and radio-protective effects.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), which include peroxides, superoxides, hydroxyl radicals and nitrous oxide, are byproducts of cellular metabolism in living organisms and are key contributors to oxidative cellular damage. Intense oxidative stress can cause cell damage and death, leading to various health issues, such as atherosclerosis, hypertension, inflammatory response syndrome, aging, respiratory syndromes, liver diseases and cancer. Plants used in traditional medicine contain a wide array of compounds capable of treating both chronic and infectious diseases. Whether used as medicines or dietary supplements, plants play a significant role in maintaining health and preventing diseases related to oxidative stress, such as cancer, atherosclerosis, diabetes, inflammation and aging.

Cymbidium aloifolium, commonly known as the aloe-leafed cymbidium, is a species of orchid found in various parts of Asia. This orchid typically grows in tropical and subtropical forests, often at elevations ranging from 300 to 2000 meters above sea level. It thrives in both epiphytic and lithophytic conditions, often found clinging to trees or rocks in its natural habitat. The phytochemical composition of *C.*

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aloifolium has a rich array of bioactive compounds. The plant is known to contain various secondary metabolites, including alkaloids, phenolics, flavonoids, saponins, tannins and glycosides. Alkaloids such as hordenine and cymbidine have been identified in *C. aloifolium*. These compounds are known for their antimicrobial and analgesic properties (Shubha and Srinivas, 2016). *C. aloifolium* is known for its diverse bioactivities also includes antimicrobial (Sujin *et al*., 2021), antioxidant, anti-inflammatory and anticancer properties (Kumar *et al*., 2022).

MATERIALS AND METHODS

The plant material (leaves) from *C. aloifolium* (Orchidaceae) was collected in April 2023 from natural forest vegetation of Paderu, Visakhapatnam, Andhra Pradesh, India (17°59′53.79″ N, 82°29′15.59″ E, elevation 1033 m). Herbarium specimens were submitted with herbarium number AUV 25556 in the Andhra University Herbarium (AUV).

The bark of the four plants was collected, cleaned, air dried and then grinded into a fine powder using an electric blender. Powdered bark materials were extracted with three different solvents, acetone, methanol and water by using a hot continuous soxhlet extraction. Preliminary phytochemical screening of plants was carried out following the standard procedures.

The bacterial pathogens utilized in this study were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), located at IMTECH in Chandigarh, India. Microorganisms tested for anti-microbial activities three Gram '+' bacteria *Bacillus subtilis* (MTCC 96), *Streptococcus mutans* (MTCC 497) and *Staphylococcus aureus* (MTCC 96) and one Gram '-' bacteria *Pseudomonas fluorescens* (MTCC 664) were cultured in nutrient agar medium, at 37°C for 24 h. Streptomycin was used as positive control and DMSO was used as negative control.

The antibacterial efficacy of the test compounds against bacterial strains was assessed using the cup plate agar diffusion method. Nutrient agar medium was prepared and poured into Petri plates and allowed to solidify under laminar airflow. The bacterial inoculum (100 µl) was inoculated from 18-hour-old cultures of the test organisms into agar well petri plates. After inoculation, five wells each of 5 mm diameter were carefully made in each plate at equal distances. Stock solutions of the test compounds (methanol, acetone, benzene and aqueous extracts of *C. aloifolium* leaves) were prepared at concentrations of 10, 5, and 2.5 mg. One well was loaded with the standard drug streptomycin in each plate. The prepared Petri dishes were incubated in a BOD incubator for 24 h at 37°C. After incubation, the zones of inhibition were measured to assess the antibacterial activity. Each experiment was repeated three times.

A stock solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) was meticulously prepared at a concentration of 0.1 mm in methanol and stored under light-protected conditions at room temperature to prevent undesired oxidation. The assay, as per standard protocols, involved the dissolution of plant extracts in methanol to achieve concentrations ranging from 20 to 120 µg/ml in separate test tubes. Subsequently, 3.0 ml of DPPH was added to each extract, ensuring thorough mixing, followed by a 30-minute incubation period in darkness. After this incubation period, the absorbance of each solution was measured at 517 nm using a UV-visible spectrophotometer (Agilent). In parallel, standard ascorbic acid solutions (20, 40, 60, 80, 100, 120 µg/ml) were subjected to the same protocol. Control samples, consisting of 1.0 ml methanol with 3.0 ml of DPPH solution, were incorporated, and methanol alone was used as the blank. All experiments were conducted in triplicate to ensure reliability. The inhibition percentage of the DPPH free radical was calculated using the following equation:

% inhibition of DPPH = [(Absorbance of control - Absorbance of test sample)/ (Absorbance of control)] x 100

The concentration (µg/ml) of the plant extract was plotted against the percentage inhibition and the IC_{50} was calculated from the intercept of linear regression obtained from the graph.

The analysis of the extracts was conducted using the Agilent Technologies GCMS, consisting of the GC-8890 and GC/MS 5977 MSD components. For injection, a split mode was employed with an 18 ml/min split flow and a purge flow of 3 ml/min. The oven's temperature underwent a programmed progression, initiating at 75°C and gradually reaching a maximum of 360°C. Two distinct

columns, namely, Polar Columns (DB-WAX) and HP-5 MS UI, were utilized with a flow rate set at 1.21 ml/min, employing helium gas (99.99%) as the carrier gas. The column temperature range was precisely controlled, spanning from 60 to 325°C. The total runtime for the GC-MS analysis was 53.5 min. Electron Impact (EI) mode was chosen for the ionization of the sample components, with an energy of 70 eV. The calculation of the relative amount of each component involved a comparison of the average peak area of each component to the overall area, providing a comprehensive assessment of the composition and abundance of the analytes in the extracts.

RESULTS AND DISCUSSION

The results of the qualitative screening conducted on the diverse *C. aloifolium* extracts revealed the presence of various primary metabolites, including carbohydrates, proteins, as well as fixed oils and fats. Additionally, secondary metabolites such as alkaloids, phenols, tannins, flavonoids, terpenoids, glycosides, cardiac glycosides, saponins and steroids were identified (Table 1). Alkaloids were present in methanol extract, and absent in acetone, aqueous, and benzene extracts. This suggested that methanol is more effective in extracting alkaloids. Quinones were absent in all extracts. Phenols were present in all extracts, with the highest levels in methanol. Acetone and aqueous extracts also showed significant phenolic content. Tannins were also present in all extracts, with the highest levels in aqueous. Flavonoids were present in methanol and acetone extracts, absent in aqueous, and present in benzene extract. Terpenoids were present in acetone, aqueous and methanol extracts. Methanol extracts exhibited the highest abundance of phytochemical compounds among all extracts, followed by acetone and aqueous extracts. In contrast, the benzene extract contained the fewest active principles compared to the other solvent extracts. It is noteworthy that phenols and tannins were consistently present in all four solvents.

Table 2 presents the antibacterial activity of different extracts [methanol (Me), acetone (Ac), water (W) and benzene (Be)] of *C. aloifolium* against four bacterial strains (*Pseudomonas fluorescens*, *Streptococcus mutans*, *Bacillus subtilis* and *Staphylococcus aureus*) at three different concentrations (10, 5 and 2.5 mg), with streptomycin as a positive control. The methanol extract exhibited moderate antibacterial activity against *S. aureus* across all concentrations, with the highest inhibition zone observed at 10 mg (18.3 ± 0.57 mm). It also showed moderate inhibition against *S. mutans* at 5 mg (16.3±0.57 mm) and *B. subtilis* at 10 mg (14.3±0.57 mm).

The acetone extract showed moderate efficacy against *P. fluorescens* with the highest inhibition at 10 mg (10±1 mm) and substantial activity against *S. mutans* at all concentrations,

Table 1. Preliminary phytochemical screening of *C. aloifolium*

Phytochemical constituents	Solvents extracts			
	Methanol	Acetone	Aqueous	Benzene
Primary metabolites				
Carbohydrates	-	-	++	-
Proteins	+	+	+	-
Fixed oils and fats	-	-	-	+
Secondary metabolites				
Alkaloids	+	-	-	-
Quinones	-	-	-	-
Phenols	++	+	+	+
Tannins	+	+	++	+
Flavonoids	++	+	+	-
Terpenoids	+	++	-	-
Glycosides	++	+	+	-
Cardiac glycosides	++	++	+	-
Saponins	++	-	+	-
Steroids	+	++	+	-
Coumarins	+	++	++	-

Where: - Absent, + Presente and ++ present in high amount.

Table 2. Antibacterial activity of different extracts (Me, Ac, Ch, W and N) at the dosages of 10, 5 and 2.5 mg

Extract	*P. fluorescens* (mg)			*Streptococcus mutans* (mg)			*Bacillus subtilis* (mg)			*Staphylococcus aureus* (mg)		
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
Me	14±1	12.5± 0.75	9.3± 0.57	16.3± 0.57	13±1	10.3± 0.57	14.3± 0.57	10±1	-	18.3± 0.57	9±1	6.3± 0.57
Ac	10±1	8.3± 0.57	-	14±1	13.3± 0.57	12.3± 0.57	12.5± 0.75	10±1	14± 0.57	-	-	-
Be	10.6± 0.57	9.3± 0.57	-	25.3± 1.21	15± 1	14.3± 0.57	15±1	12.3± 0.57	11±1	15.6± 0.57	11.3± 0.57	7±1
Aq	-	-	-	-	-	-	-	-	-	-	-	-
+ Ve	25±1.23 mm			28±0.95			25±0.57			27.6±1.52		

Data showing zone of inhibition in mm. Aq: Aqueous extract, Me: Methanol extract, Ac: Acetone extract, Be: Benzene extract. Values represent mean ± standard deviations. "-" for no zone of inhibition. A zone of inhibition with a diameter of less than 6 mm was considered inactive.

peaking at 5 mg (13.3±0.57 mm). It also effectively inhibited *B. subtilis* with zones of inhibition of 12.5±0.75 mm at 10 mg and 14±0.57 mm at 5 mg, and displayed significant activity against *S. aureus* at 10 mg (14±0.57 mm). Benzene extracts exhibited the highest antibacterial activity against *S. mutans* at all concentrations, particularly at 10 mg (25.3±1.21 mm), and were also active against *P. fluorescens* at 10 mg (10.6 ± 0.57 mm). Additionally, benzene extracts showed substantial inhibition against *S. aureus* and *B. subtilis* at 10 mg (15.6±0.57 and 15±1 mm, respectively).

In contrast, the aqueous extract did not exhibit significant antibacterial activity against any of the tested bacterial strains at any concentration. The positive control, streptomycin, exhibited substantial inhibition zones against all four bacterial strains, validating the experimental setup and serving as a benchmark for antibacterial activity (25±1.23 mm for *P. fluorescens*, 28±0.95 mm for *S. mutans*, 25±0.57 mm for *B. subtilis* and 27.6±1.52 mm for *S. aureus*).

The antimicrobial activity of *C. aloifolium* extracts was evaluated against 10 clinical pathogenic bacteria using various solvents. The chloroform extract was the most effective, showing significant inhibition zones ranging from 7-18 mm, especially against *S. aureus* at a 500 µg/ml concentration. The hexane extract had the least effect, with only a few bacteria being susceptible. Chloroform extracts were more potent than methanol and hexane extracts, particularly at concentrations of 100, 250 and 500 mg/ml (Sujin *et al*., 2021). The aqueous extract of *C. aloifolium* showed the highest inhibition against *Rhizopus* sp. followed by *Aspergillus niger* and *S. aureus*. Methanol and ethanol extracts were also active against *Staphylococcus aureus* and *Klebsiella pneumoniae*. Acetone extract was effective against *B. subtilis* but not against *Escherichia coli*, *Klebsiella pneumoniae* and *S. aureus*. Aqueous extracts performed better than the positive control, with methanol and acetone showing the least inhibitory activity (Soumiya and Williams, 2017).

Methanol and acetone extracts of *C. aloifolium* demonstrated the highest antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*. The extracts were further evaluated for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), with values ranging from 0.12 to 3.75 mg/ml against the tested pathogens (Shubha and Srinivas, 2016). *C. aloifolium* exhibited significant antimicrobial properties, with chloroform and aqueous extracts showing the highest potency against a range of pathogens. This suggests potential for the development of new antimicrobial agents derived from *C. aloifolium*, warranting further research into their mechanisms of action and therapeutic applications.

Anti-oxidant activity examined the activity of aqueous, acetone, benzene and methanol extracts of *C. aloifolium* leaves using the DPPH assay. The results demonstrated that various solvent extracts impacted the DPPH radical, with activities compared to ascorbic acid, a known antioxidant. The radical scavenging activity (RSA) of aqueous extracts increased steadily from 21.14% at 20 µg/ml to 77.52% at 120 µg/ml, with low standard deviations

indicating consistent measurements. This extract showed a moderate RSA, significantly increasing in activity, particularly from 80 µg/ml onwards. The RSA of acetone extracts increased from 34.07% at 20 µg/ml to 75.42% at 120 µg/ml. The RSA of methanol extract increased from 36.76% at 20 µg/ml to 91.25% at 120 µg/ml. This extract exhibited the highest RSA among all extracts at 120 µg/ml, approaching the activity of ascorbic acid. The methanol extract showed a consistently high RSA, indicating strong antioxidant properties. The RSA of benzene extract increased from 33.68% at 20 µg/ml to 81.25% at 120 µg/ml. The benzene extract showed significant RSA, particularly at higher concentrations, though slightly lower than the methanol extract. The consistency of measurements was good, with low standard deviations (Fig. 1).

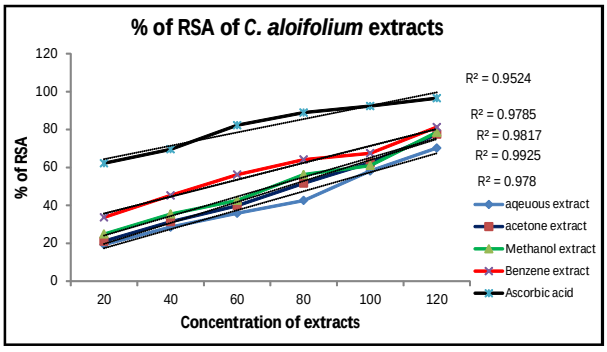

Fig. 1. Percentage of RSA in DPPH activity of *C. aloifolium.*

Ascorbic acid, a standard antioxidant, showed the highest RSA at all concentrations, starting from 62.23% at 20 µg/ml to 96.54% at 120 µg/ml. The high RSA values and low standard deviations highlight the potent and consistent antioxidant activity of ascorbic acid. Among the extracts tested, the methanol extract demonstrated the highest RSA, closely followed by the benzene extract, indicating that these solvents were particularly effective in extracting antioxidant compounds from *C. aloifolium*. All extracts showed a concentration-dependent increase in RSA, typical for antioxidant activity assays. The methanol extract, in particular, showed a sharp increase in activity with increasing concentration.

To interpret the results from the DPPH method, IC_{50} values were calculated from the slope and intercepts of the linear regression equation from RSA% values. IC_{50} values are defined as the concentration of substrate that causes a 50% loss of the DPPH activity. The lower the IC_{50} value, the higher the antioxidant activity.The methanolic extract demonstrated the highest antioxidant activity, with an IC_{50} value of 20.45±1.02 µg/ml, whereas the acetone extract showed the lowest antioxidant activity, with an IC_{50} value of 74.82±1.64 µg/ml (Fig. 2). The effect of solvent influenced the scavenging capacity of plant extracts because of the presence of polyhydroxy or polyphenolic compounds. Methanolic extracts exhibited greater radical scavenging activity than acetone and aqueous extracts due to the extraction of polyhydroxy chemicals such as phenolic acids and flavonoids (Kiran *et al*., 2024). Methanol, a polar solvent miscible with water, effectively extracted secondary metabolites. Acetone, an intermediate polar solvent, generally extracted medium polar compounds, while aqueous solvent, the most polar, was used to extract a wide range of polar compounds (Lee *et al*., 2024).

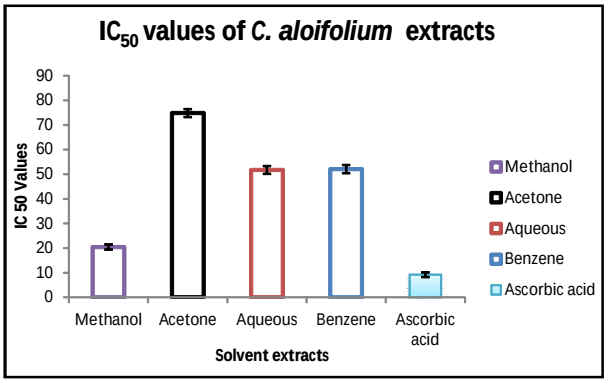

Fig. 2. IC_{50} values of *C. aloifolium* extracts.

The experimental procedure outlined in this study led to the identification of numerous peaks in the Gas Chromatography-Mass Spectrometry (GC-MS) data obtained from the methanol extract of *C. aloifolium*. These peaks indicated the presence of nine different chemical compounds (Fig. 3). Subsequently, these compounds are thoroughly documented in Table 3, which include details such as peak area percentages, molecular formulas, molecular weights, and their order based on retention times. A comprehensive literature review was conducted to explore the biological activities associated with these compounds, revealing that most possessed diverse pharmacological and therapeutic properties. These compounds were classified into various categories, including terpenes, fatty acids,

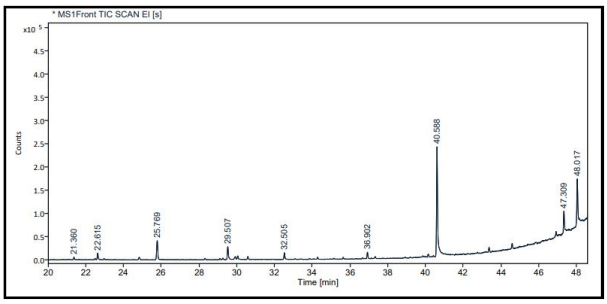

Fig. 3. GC-MS chromatogram of methanol leaf extract of *C. aloifolium*.

flavones, phenols, steroids and other substances. Notably, the identified compounds exhibited a wide range of bioactive effects, including anti-cancer, antimicrobial, anti-inflammatory, sedative, anti-asthma, analgesic, antioxidant and pain-relieving properties.

The compounds within the dataset were analyzed for their respective peak area percentages, revealing the prominence of certain components. Notably, 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (49.19%) and γ-Sitosterol (19.89%) constituted the highest significance in the dataset and were the major compounds in this leaf methanol extract. Other significant compounds included Cholesta-22,24-dien-5-ol, 4,4-dimethyl (9.17%), n-Hexadecanoic acid (8.16%) and Phytol (5.49%). Additional noteworthy compounds were 2-Piperidinone, N-[4-bromo-n-butyl]- (2.59%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (2.38%) and 2-Pentadecanone, 6,10,14-trimethyl (2.35%). One minor constituent, constituting less than 0.9% of the dataset, was (S, E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone (0.77%).

The methanol extract of *C. aloifolium* fruit contained a variety of phytochemical compounds with diverse bioactivities, including antioxidant, antimicrobial, anti-inflammatory and cholesterol-lowering properties. 2-Pentadecanone, 6,10,14-trimethyl is a ketone derivative that possessed antimicrobial, antifungal and antioxidant properties and may have applications in the fragrance and flavour industries. Limited information is available about (S, E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone, but cyclohexenone derivatives have been studied for antioxidant, anti-inflammatory and anticancer properties.

n-Hexadecanoic acid (palmitic acid) is a saturated fatty acid with antimicrobial and anti-inflammatory properties. Excessive intake is linked to adverse health effects such as insulin resistance and cardiovascular diseases (Najda *et al*., 2021). Phytol is a diterpene alcohol with potential antioxidant, anti-inflammatory and anticancer activities. Phytol derivatives have also been investigated for neuroprotective effects (Islam *et al*., 2018). 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (DEHP) is a phthalate ester used as a plasticizer with endocrine-disrupting effects and potential reproductive toxicity, carcinogenicity and it is a strong immunomodulatory B-cell stimulant (Javed *et al*., 2022). A sterol derivative Cholesta-22,24-dien-5-ol, 4,4-dimethyl involved in various biological processes, essential for cell membranes, and potentially has cholesterol-lowering effects. A plant sterol γ-Sitosterol with cholesterol-lowering, anti-inflammatory and antioxidant properties is promising for the

Table 3. Phytochemical compounds reported in leaf methanol extract of *C. aloifolium*

S. No.	Compound name	Rt minute	Mol. weight (g/mol)	Mol. formula	Area (%)
1.	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	21.360	222.28	$C_{13}H_{18}O_3$	0.77
2.	2-Pentadecanone, 6,10,14-trimethyl	22.615	268.5	$C_{18}H_{36}O$	2.35
3.	n-Hexadecanoic acid	25.769	256.42	$C_{16}H_{32}O_2$	8.16
4.	Phytol	29.507	296.5	$C_{20}H_{40}O$	5.49
5.	2-Piperidinone, N-[4-bromo-n-butyl]-	32.505	234.13	$C_9H_{16}BrNO$	2.59
6.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	36.902	330.5	$C_{19}H_{38}O_4$	2.38
7.	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	40.588	390.6	$C_{24}H_{38}O_4$	49.19
8.	Cholesta-22,24-dien-5-ol, 4,4-dimethyl	47.309	412.7	$C_{29}H_{48}O$	9.17
9.	γ-Sitosterol	48.017	414.7067	$C_{29}H_{50}O$	19.89

prevention and treatment of cardiovascular diseases and cancer (Huifen *et al*., 2021).

CONCLUSION

The study investigated the bioactivities of various solvent extracts from the leaf of *C. aloifolium*, focusing on phytochemical screening, antimicrobial activity, antioxidant activity and the identification of compounds through Gas Chromatography-Mass Spectrometry (GC-MS). Methanol extracts exhibited moderate antibacterial activity, particularly against *S. aureus* and *S. mutans*. Benzene extracts showed the highest antibacterial activity, especially against *S. mutans* and *P. fluorescens*. In terms of antioxidant activity, methanol extracts demonstrated the highest radical scavenging activity (RSA), closely followed by benzene extracts. GC-MS analysis of the methanol extract identified nine different chemical compounds with significant bioactive properties. Major compounds identified included 13-Benzenedicarboxylic acid bis(2-ethylhexyl) ester, γ-Sitosterol, Cholesta-22,24-dien-5-ol, n-Hexadecanoic acid and phytol. These compounds were associated with diverse pharmacological and therapeutic properties, such as anti-cancer, antimicrobial, anti-inflammatory, sedative, analgesic and antioxidant effects. Among the various solvents used, methanol proved to be the most effective in extracting a wide range of phytochemicals, which contributed to its high antioxidant activity. The GC-MS analysis further revealed the presence of several compounds with promising pharmacological activities. These findings suggest that *C. aloifolium* could be a valuable resource for developing natural therapeutic agents.

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