International Journal of Secondary Metabolite
2025, Vol. 12, No. 1, 33–45
https://doi.org/10.21448/ijsm.1505737
journal homepage: https://dergipark.org.tr/en/pub/ijsm
Research Article

Comprehensive analysis of antibacterial and antioxidant properties in *Calotropis gigantean* (Apocynaceae) leaf extracts

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ARTICLE HISTORY
Received: June 28, 2024
Accepted: Aug. 03, 2024

KEYWORDS
Calotropis gigantea,
Leaf extract,
Antibacterial,
Antioxidant,
LC-MS.

Abstract: *Calotropis gigantea* (L.) W.T.Aiton., commonly known as arka, is a wild tropical plant from the Apocynaceae family with various medicinal properties. Previous studies have stated its antioxidant and antibacterial properties. For this purpose, leaf extracts of *C. gigantea* were prepared using a variety of solvents: ethanol, methanol, and n-hexane. For phytochemical bioactive compounds identification LC-MS analysis was performed, and to evaluate antibacterial activity against different gram positive (*Bacillus subtilis*, *Bacillus muralis*) and gram negative (*E. coli, Acetobactor rhizospherensis*) bacterial strains well diffusion method was applied. Antioxidant activity was observed using ABTS (2,2′-azino-bis-(3-ethylenebenzothiazoline)-6-sulfonic acid assay) and DPPH (2,2-diphenylpicrylhydrazyl assa) assays. The LC-MS (liquid chromatography-mass spectrometry) analysis revealed that ethanol leaf extract contained the highest number of compounds (14), while a 50% ethanol-methanol mixture had the fewest. In antimicrobial activity tests, methanol leaf extract exhibited the greatest inhibition zone against Bacillus subtilis (8 mm), and n-hexane the smallest (2 mm). Ethanol leaf extract had the highest inhibition against E. coli (9.5 mm), with methanol showing the lowest (4 mm). For *Acetobacter rhizopherensis*, ethanol extract demonstrated the largest inhibition zone (7.75 mm), while n-hexane showed the smallest (6 mm). Against *Bacillus muralis*, n-hexane showed the highest inhibition (5.75 mm), and methanol the lowest (3.5 mm). The ABTS assay indicated that ethanol extract had the highest inhibition activity (9.73%), and n-hexane the lowest (5.64%). The DPPH assay revealed that methanol extract had the highest radical remaining activity (99.68%), with n-hexane having the lowest (97.19%). *C. gigantea* is a prospective source of antioxidant and antibacterial agent.

1. INTRODUCTION

C. gigantea is a prevalent plant species found in barren and uncultivated areas, and it is usually referred to as gigantic milkweed (see Figure 1). This plant is indigenous to Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippines, Thailand, and Sri Lanka. The plant

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e-ISSN: 2148-6905

has ovate, pale green foliage, a latex-filled stem, and clusters of glossy blooms that may be either white or lavender in hue. *C. gigantea* is readily accessible in India and is used for several therapeutic reasons in traditional medicine (Kumar *et al.*, 2012). Leaf of the *C. gigantea* are significantly known to have antibacterial and antioxidants activities. *C. gigantea* and *Calotropis procera* (Aiton) W.T.Aiton are the two species of *Calotropis* that are now in existence. In the past, these plants were mostly employed as ayurvedic medicines, and they were known by the common names "Sweet Arka" and "Raktha Arka," respectively. The botanical characteristics and pharmacological actions of these plants are identical (Bhatia *et al.*, 2022). The taxonomical classification of the plant *Calotropis gigantea* is described in the (Table 1).

Figure 1. General view photos of *Calotropis gigantea.*

Table 1. Taxonomical classification of *C. gigantea.*

Kingdom	Plantae
Phylum	Tracheophyte
Sub-phylum	Euphyllophyte
Class	Magnoliopsida
Sub-class	Asteridae
Order	Gentianales
Family	Apocynaceae
Subfamily	Asclepiadoideae
Tribe	Asclepiadeae
Genus	*Calotropis* R.Br.
Species	*Calotropis gigantea* (L.) W.T.Aiton

1.1. Vernacular Names

In many nations throughout the world, *Calotropis gigantea* is known by the following common names (Mahajan & Badgujar, 2008; Tiwari *et al.*, 2014). That was displayed in Table 2. *C. gigantea* can reach heights of 8–10 feet (Choudhary *et al.*, 2013). This plant contains clusters of waxy white or lavender blooms, oval light green leaves, and a milky latex stem. In India, *C. gigantea* is frequently found and used as medicine for a variety of illnesses (Alam *et al.*, 2008). The *Calotropis* plant has different names in different countries, as shown in Table 2 (Sharma & Tripathi, 2009).

Madagascar, Asia (including India, Pakistan, and Afghanistan), and tropical and subtropical Africa all have it (Somalia, Egypt, Libya, south Algeria, Morocco, Mauritania, and Senegal). Jordan, India, Iran, Arabia, Southern Asia, and Indochina (Rajamohan *et al.*, 2014; Sharma *et al.*, 2016). The plant has naturally grown on islands in Australia, including those in Indonesia and the Pacific, the Caribbean, and South and Central America (Rajamohan *et al.*, 2014).

Table 2. Vernacular names of the *C. gigantea* in the world.

Names of countries	Common name of *Calotropis gigantea* .
India	In Sanskrit it is called as Arka, Vasuki, Alarka, pratapass. Safed aak, Aak, Alarkh, Madar, Sveta Arka, Akanda, and Bara Akand are all used in Hindi.
Malaysia	In Malaysia, it is called Rembega, Remiges, and kemengu.
English countries	In almost all English countries it is called Giant milkweed, a Crown flower.
Indonesia	In Indonesia it is called as Sidaguri in Javanese, Rubik in Aceh.
Thailand	Paan-theun and Po-theun are commonly used.
Laos	Dok kap, Dok hak and Kok are mostly used.
French	Faux Arbre and Mercure vegetal are common names in French countries.

Calotropis may grow wild up to 900 meters across the nation and is very tolerant to salt and drought (Rani *et al.,* 2019). When dried, the milky latex found in the stem of *C. gigantea* serves as an antinode for the venom (snake poison). Its dried leaves are anti-inflammatory and are used to make cough medication and to cure paralysis (Al-Maskri *et al.,* 2011; Gyawali *et al.,* 2020; Muhammad *et al.,* 2011; Shahzadi *et al.,* 2017). *Calotropis* is regarded as an aromatic herb with therapeutic properties. The latex of the *Calotropis* plant contains cardiac toxins (Shahzadi *et al.,* 2017). The *Calotropis* plant's stems and leaves also contain essential oil, which is discovered through GC-MS (gas chromatography-mass spectrometry) analysis. (Merzaia *et al.,* 2017; Shahzadi *et al.,* 2017; Tiwari *et al.,* 2014). Many parts of the *Calotropis* plant, including the leaves, stem flowers, and root barks, are used in medicine to treat illnesses that are frequently found in people, including fever, arthritis, digestion issues, cough, loose stools, and nausea (Patel *et al.,* 2014).

The aim of the current research was to evaluate the antioxidant and antibacterial activities *of C. gigantea* leaf extract. It was meticulously examined for a variety of therapeutic uses, including the utilization of the flowers for cytotoxic, analgesic, and antibacterial properties (Choudhary *et al.,* 2013; Pathak & Argal, 2007). Plant leaves and other internal components have been employed for their antibacterial, antifungal, and antidiarrheal properties (Chitme *et al.,* 2004; Habib & Karim, 2009; Kumar *et al.,* 2010a). *Calotropis* roots have been utilized for CNS activity, wound healing, cytotoxicity, antibacterial, and antipyretic effects (Alam *et al.,* 2009; Alam *et al.,* 2008; Argal & Pathak, 2006; Chitme *et al.,* 2005; Deshmukh *et al.,* 2009; Namrata *et al.,* 2010; Wang *et al.,* 2008). This plant's latex was employed for its procoagulant, antibacterial, and wound-healing properties (Kumar *et al.,* 2010b; Lodhi *et al.,* 2009; Nalwaya *et al.,* 2009; Rajesh *et al.,* 2005). The stem of the plant was employed as a hepatoprotective agent(Sivapalan *et al.,* 2023).

2. MATERIAL and METHODS

2.1. Extraction of the Plant Material

Calotropis gigantea fully matured leaves of 10 biological replicates and three technical replicates were collected from the botanical garden, Government College University Lahore in May 2022, washed with distilled water and allowed to dry (in shade) for a week. Leaves were ground to fine powder by using a pistil and motor. Extraction was done by dissolving 5 g of leaf powder in 10 mL of various types of solvents like ethanol, methanol, 50% (ethanol and methanol), and n-hexane. 5g of powdered leaves were dissolved in 10 mL of aforementioned solvents and allowed to stand for 36 hrs. Each type of extract was filtered out by using simple filtration method. And then, these solutions were examined for antibacterial and antioxidant activities (Yesmin *et al.,* 2008).

2.2. Antibacterial Activity

Fully characterized bacterial strains *Bacillus subtilis*, *Bacillus muralis*, *E. coli*, *Acetobacter*

rhizospherensis were purchased from the microbiology lab of COMSTS University Islamabad, Abbottabad Campus. Antimicrobial agents are biological and chemical substances that inhibit the development of microorganisms and aid in their eradication. It is necessary to conduct an antimicrobial analysis on the pure separated ingredient from a natural source or the crude extract of the plant to ascertain their effectiveness against various pathogenic organism types. The well-diffusion method was employed to evaluate the anti-bacterial effect of *C. gigantea* leaf extract. Gram-positive (*Bacillus subtilis*, *Bacillus muralis*) and gram-negative (*E. coli*, *Acetobacter rhizospherensis*) bacterial strains were utilized as test organisms (Mandal *et al.*, 2022).

2.3. Well-Diffusion Method

Agar solution was prepared: 1.25g yeast, 2.5g NaCl, 2.5g tryptone, and 3.75g Agar were added in 250 mL of distilled water. Maintained the pH of the solution at 7 by adding a few drops of NaOH. A clear solution was obtained accompanied by stirring and covered with aluminum foil. Petri plates, Agar solution, micro-tips (blue and yellow), inoculating loops, glass spreaders and solvents (distilled water and DMSO) were autoclaved for sterilization. The agar media was spread on petri plates, and the plates were then allowed to dry. On the solidified agar media freshly grown bacterial strains were applied using a glass spreader. Yellow microtips were used to create wells in the agar diffused plates. Then, using a micropipette, a 1 mg/mL solution of the crude extract of *C. gigantea* leaves in DMSO was injected into the wells. For positive control, 1 mg/1mL ampicillin solution in distilled water was utilized. DMSO was used as a negative control. To test the samples' antibacterial activity, all the samples were injected through micropipettes into the wells of the petri plates, covered the plates, and left for 24 hours (Julius *et al.*, 2021).

2.4. Antioxidant Activity

2.4.1. *ABTS assay*

7 mM ABTS and 2.45 mM potassium persulfate solutions were prepared in distilled water. 7.5 mL of ABTS solution was added to 2.5 mL of potassium persulfate solution in a test tube and left for 24 hours in the dark. this radical cation ABTS+ was used to check the antioxidant activity of the sample. The absorbance of stock solution was maintained between (0.709 - 0.693) at 734 nm. 2.5 mL of ABTS solution was added to different test tubes, and then 10 µL of each sample was added to determine the antioxidant activity of *C. gigantea* leaves extract. Test tubes were placed in the dark for 8 minutes. UV/Vis spectroscopy was used to observe(Srivastava *et al.*, 2020).

2.4.2. *DPPH assay*

DPPH solution was prepared using 25 mg/L in methanol, and methanol was used to dilute it until absorbance was maintained at 0.963 (λ_{max} =763nm). After adding 2.5 mL of the stock solution to several test tubes, 10µL of samples were mixed in it. Test tubes were placed in dark for 30 minutes, and the change in absorbance was measured(Sangeetha *et al.*, 2020).

3. FINDINGS

3.1 LC-MS Analysis

3.1.1. *Phytochemical screening of leaves of C. gigantea*

Using a Bruker Dionex Ultimate 3000 quadrupole time-of-flight mass spectrometer, the chemicals in all of the *C. gigantea* samples were identified. Water containing 0.1% formic acid (A) and acetonitrile served as the eluents. A flow rate of 0.4 mL/min was used to complete the gradient programme over the course of 30 minutes. The injection volume for the sample was 2 L. The Bruker Dionex ultimate 3000 qTOF mass spectrometer, which has electrospray ionization in both positive and negative modes, was used to conduct the qualitative analysis. As a nebulizing, collision, and drying gas, nitrogen was utilized.

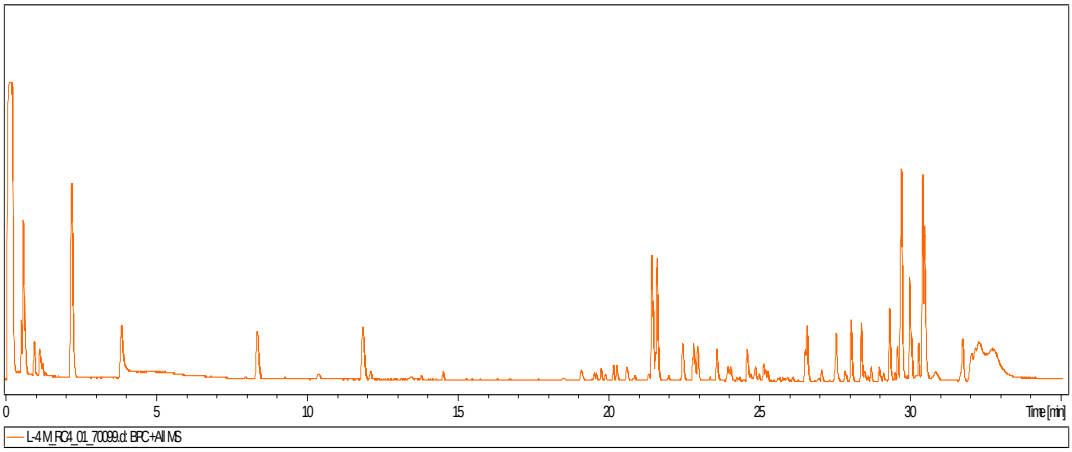
3.1.2. *LC-MS analysis of methanolic extract*

The studies on the active principle of LC-MS analysis of methanol extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in the Table 3, Figure 2.

Table 3. Different types of compounds and their retention time in MeOH leaves extract.

Sr. No	Retention Time	Compound	Molecular weight
1	2.2	4-Heptylobenzoic acid	237.145
2	3.9	Oxypeucedanin	287.092
3	11.9	2,4,6-Triphenyl-1-hexene	313.194
4	21.4	Hexadecasphinganine	274.274
5	23.0	Phytosphingosine	318.299
6	26.6	Monoethylhexyl phthalic acid	301.140
7	27.5	Acetyl tributyl citrate	425.213
8	28.4	Oleamide	282.279

Figure 2. Graphical representation of different compounds in methanol leaf extract.



3.1.3. *LC-MS analysis of ethanolic extract*

The studies on the active principle of LC-MS analysis of ethanol extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 4, Figure 3.

Figure 3. Graphical representation of different compounds in ethanol leaves extract.

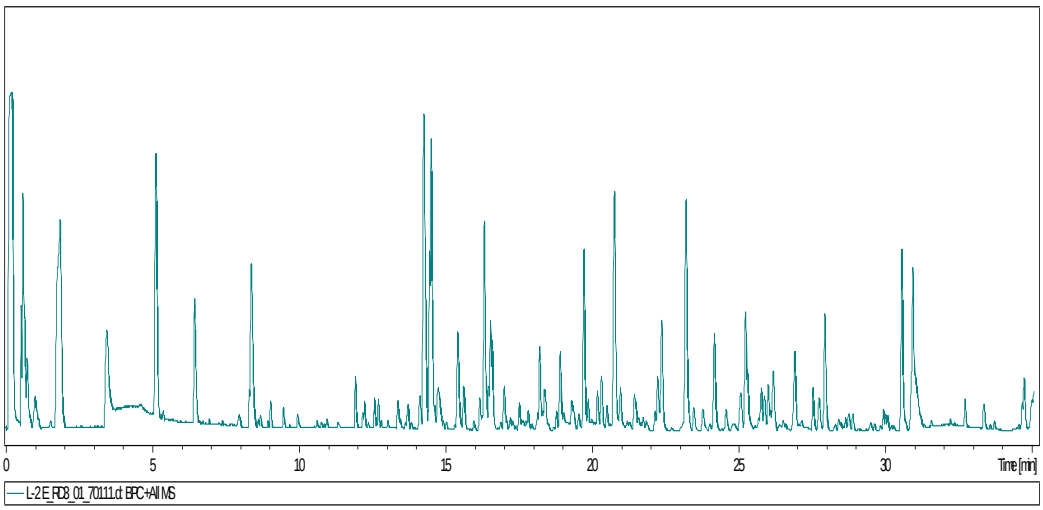


Table 4. Different types of compounds and their retention time in EtOH leaves extract.

Sr. No	Retention Time	Compound	Molecular weight
1	5.2	Ethofumesate	287.092
2	11.9	Calactin	533.273
3	12.6	4,4-Difluoropregn-5ene-3,20-dione	351.212
4	13.4	Stearidonic acid	277.215
5	13.8	6-Sgofaol	277.179
6	14.3	Hexadecasphinganine	274.275
7	17.0	Resolvin E2	357.202
8	18.9	13-cis-Retinoic acid	301.216
9	19.7	All-trans-4-Oxoretinoic acid	315.195
10	20.8	Resolvin E2	357.202
11	23.2	13-cis-Retinoic acid	323.197
12	25.2	Icosapentaemoic acid	303.230
13	25.8	Metholone.	327.228
14	26.9	Arachidonic acid	305.247
15	27.9	Octadecanamide	284.294
16	30.9	Bis(2-ethylhexyl) phthalate.	413.264

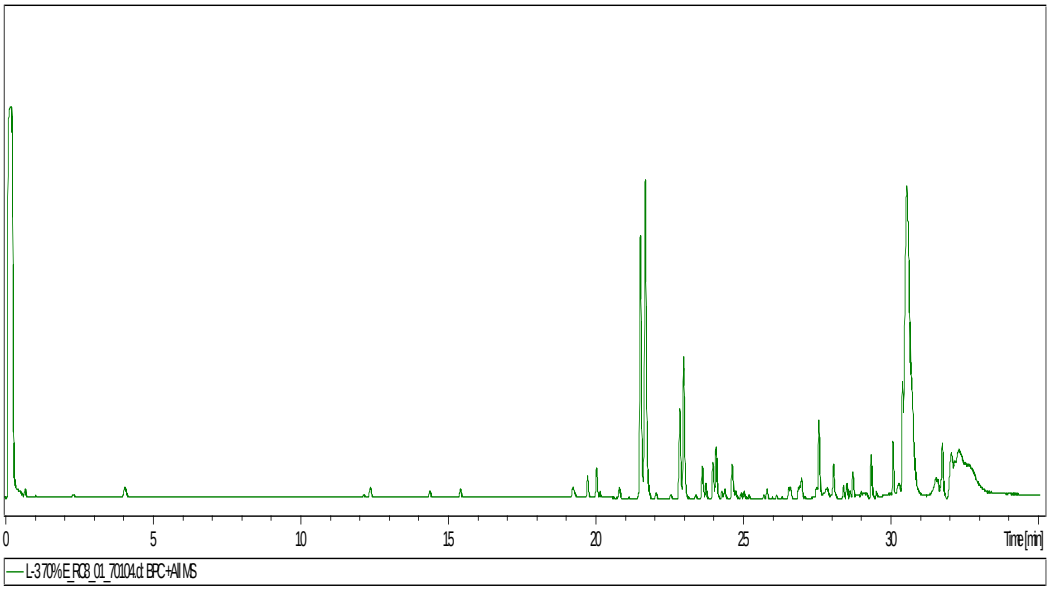
3.1.4. *LC-MS analysis of n-hexane extract*

The studies on the active principle of LC-MS analysis of n-hexane extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 5, Figure 4.

Table 5. Different types of compounds and their retention time in n-hexane leaf extract.

Sr. No	Retention Time	Compound	Molecular weight
1	21.5	Hexadecasphinganine	274.274
2	22.8	Sphinganine	302.305
3	23.0	Phytosphingosine	318.300
4	27.5	Acetyl tributyl citrate	425.213
5	30.1	Cepharanthine	607.291

Figure 4. Graphical representation of different compounds in n-hexane leaf extract.



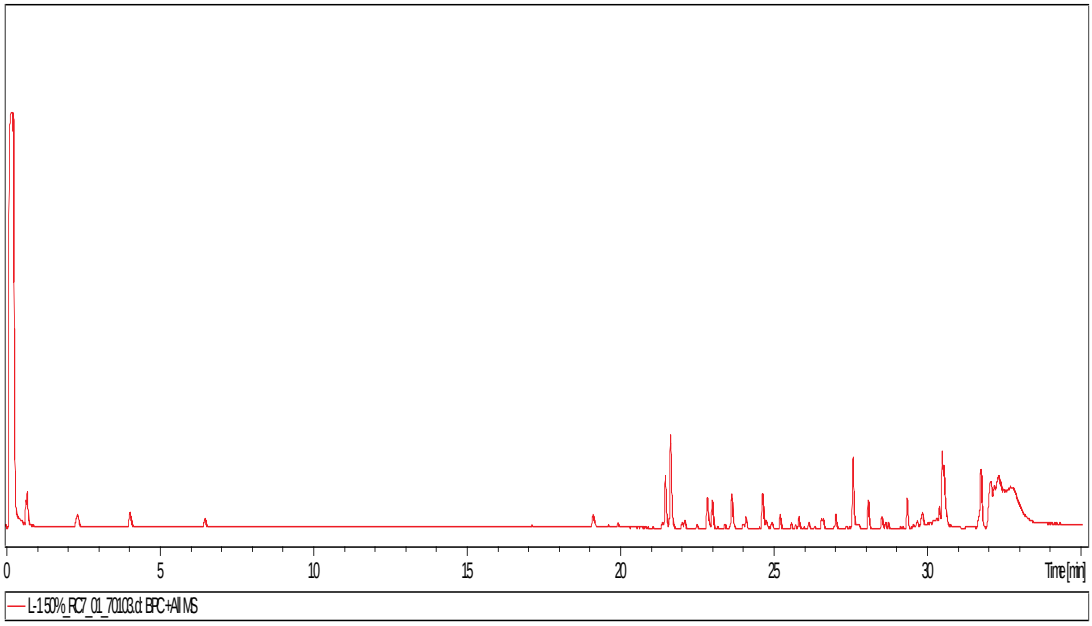
3.1.5. *LC-MS analysis of 50% (Methanol + Ethanol) extract*

The studies on the active principle of LC-MS analysis of 50%(methanol-ethanol) extract of leaves of the plant clearly showed the presence of different compounds. The active principles with their Retention time (RT), Compound names, Molecular formula (MF), and Parent ion that showed the molecular weight of that compound are shown in Table 6, Figure 5.

Table 6. Different compoaunds and their retention time in 50% (MeOH-EtOH) leaf extract.

Sr. No	Retention Time	Compound	Molecular weight
1	25.2	All-trans-4-Oxoretinoic acid	315.194
2	25.8	Prostaglandin J2	357.202
3	27.0	4,5-Leukotriene A4	341.207

Figure 5. Graphical representation of different compounds in 50% (MeOH-EtOH) leaf extract.



3.2. Antibacterial Activity

After 24 hours, the antibacterial activity of the *C. gigantea* leaf extract against the bacterial strains is observed in each well of the agar-diffused medium.

3.2.1. *Zone of inhibition of antibacterial activity*

The zone of inhibition was calculated for each sample, and the extract of *C. gigantea* leaves showed a different zone of inhibition for each type of bacteria that we utilized, which are displayed in the Table 7, Figure 6, Figure 7. Sample representations applied on the petridishes is shown in Table 8.

Table 7. Petri plates various signs.

Signs	Extracts
1	Ethanol leaves extract
2	Methanol leaves extract
3	n-hexane leaves extract
(+)	positive control
(-)	negative control

Table 8. Calculated zone of inhibition against various bacterial strains.

Bacteria	Sample	Zone of inhibition
Bacillus-muralis	Ethanol leaves extract	4.75 mm
Bacillus-muralis	Methanol leaves extract	3.5 mm
Bacillus-muralis	n-hexane leaves extract	5.75 mm
Bacillus-muralis	+ve control	6.5 mm
Bacillus-muralis	-ve control	3.5 mm
Acetobacter-rhizospheres	Ethanol leaves extract	7.75mm
Acetobacter-rhizospheres	Methanol leaves extract	4.25 mm
Acetobacter-rhizospheres	n-hexane leaves extract	6 mm
Acetobacter-rhizospheres	+ve control	6.5mm
Acetobacter-rhizospheres	-ve control	2 mm
E. coli	Ethanol leaves extract	9.5mm
E. coli	Methanol leaves extract	4mm
E. coli	n-hexane leaves extract	8mm
E. coli	+ve control	1.5mm
E. coli	-ve control	4.25mm
Bacillus-subtilis	Ethanol leaves extract	3.75 mm
Bacillus-subtilis	Methanol leaves extract	8.5 mm
Bacillus-subtilis	n-hexane leaves extract	2 mm
Bacillus-subtilis	+ve control	12 mm
Bacillus-subtilis	-ve control	No activity

Figure 6. Zone of inhibition shown against *B. subtilis* and *A. rhizospheres.*

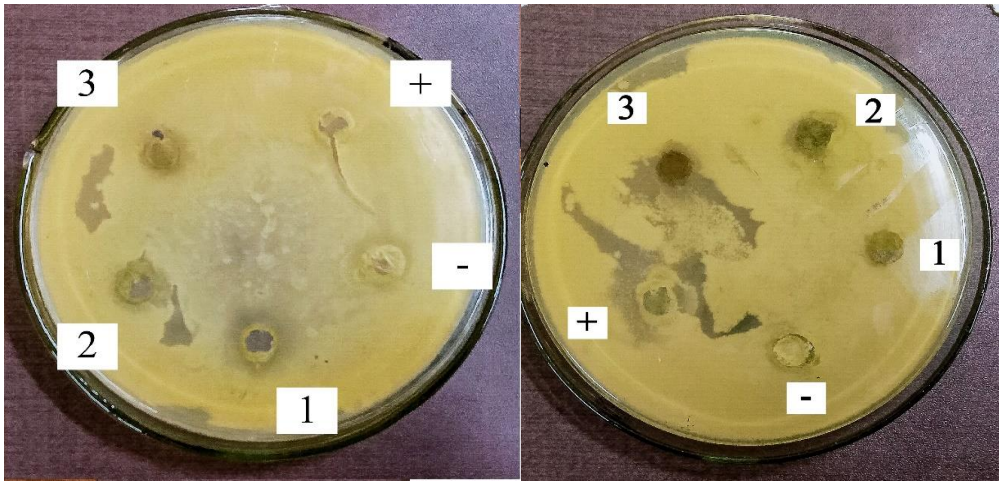
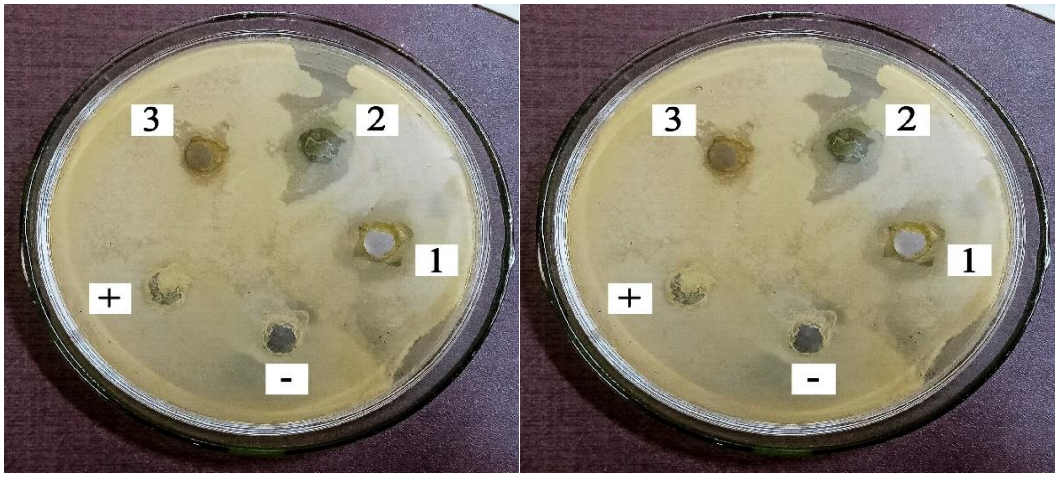


Figure 7. Zone of inhibition shown against *E. coli* and *B. muralis.*



3.3. Antioxidant Activity

3.3.1. *ABTS+ assay*

Reduction in the absorbance was observed and then % inhibition of each sample was calculated by using the following formula, all the values of each sample are written in Table 9.

$$\% \text{ Inhibition (734nm)} = (1-A_s/A^0) \times 100$$

A_s is the absorbance of the sample and A^0 is the absorbance of ABTS+. Here $\mathbf{A^0} = 0.709$

Table 9. % Inhibition of ABTS and absorbance of the sample are shown.

Sample	The absorbance of the sample (As)	% Inhibition of ABTS
n-hexane leaves extract	0.669	5.64
Ethanol leaves extract	0.640	9.73
Methanol leaves extract	0.654	7.75

The addition of the crude leaf extract caused the absorbance to decrease, demonstrating that it has antioxidant properties and the potential to stop oxidative damage brought on by free radical systems. MeOH, EtOH, and N-hexane leaf extract samples were compared in a comparative analysis. Better antioxidant activity was demonstrated by ethanolic leaf extract. As seen below, each extract exhibits distinct antioxidant activity when compared to one another.

Ethanol leaves extract > Methanol leaves extract > N- hexane leaves extract

3.3.2. *DPPH assay*

The change in the absorbance was measured and then the % of DPPH radical remaining was calculated using the following formula and all the data is written in the Table 10.

$$\% \text{ DPPH radical remaining} = A_S/A_o \times 100$$

A_s is the absorbance of the sample, A^0 is the absorbance of DPPH. Here $A_o = 0.963$

Table 10. %DPPH radical remaining and absorbance are shown.

Sample	The absorbance of the sample (As)	% DPPH radical remaining
n-hexane leaves extract	0.960	97.19
Ethanol leaves extract	0.959	99.68
Methanol leaves extract	0.936	99.58

When *C. gigantea* leaves extract was added to test tubes containing DPPH solution, the absorbance was found to decrease, indicating the extract's antioxidant properties. Comparative analysis between different extracts revealed that methanol leaf extract of the leaves had higher antioxidant properties. The antioxidant activity of each extract varies when compared to one another, as can be seen below.

Methanol leaves extract > Ethanol leaves extract > n-hexane leaves extract

4. DISCUSSION and CONCLUSION

The LC-MS assessment of the different solvent leaf extracts revealed substantial variation in chemical composition. The ethanol leaf extract exhibited the maximum number of compounds (14), while the 50% ethanol-methanol combination had the lowest number. The difference in the composition of compounds may directly impact the biological activities of the extracts, emphasizing the need to choose the appropriate solvent in phytochemical research.

The methanol leaf extract had the highest level of antimicrobial activity, as shown by the largest inhibition zone (8.5 mm) against *Bacillus subtilis*. This suggests that methanol is more efficient

in extracting antibacterial chemicals that specifically target this bacterium. In contrast, the n-hexane extract exhibited the lowest inhibitory zone measuring 2 mm, suggesting a lesser level of effectiveness. This discovery aligns with prior research that has shown methanol's superior efficacy as a solvent for extracting polar molecules with antibacterial characteristics (Altemimi *et al.*, 2017).

The ethanol leaf extract exhibited the greatest inhibition (9.5 mm) against *Escherichia coli*, whilst methanol showed the lowest inhibition (4 mm). This result highlights the varying effectiveness of solvents in extracting chemicals that have activity against Gram-negative bacteria such as *E. coli.* According to (Klūga *et al.*, 2021) , ethanol, due to its moderate polarity, has the ability to extract a wider variety of bioactive chemicals compared to methanol or n-hexane.

The investigation further revealed that the ethanol extract exhibited the most significant inhibitory zone against *Acetobacter rhizopherensis*, measuring 7.75 mm. Conversely, the n-hexane extract had the smallest zone, measuring 6 mm. This indicates that the chemicals that are capable of effectively combating *A. rhizopherensis* have a higher solubility in ethanol compared to n-hexane, which is a less polar solvent. This is consistent with the widely accepted notion that polar solvents are more efficient in extracting antimicrobial drugs (Borah *et al.*, 2020).

N-hexane exhibited the greatest inhibition (5.75 mm) for *Bacillus muralis*, whilst methanol had the lowest inhibition (3.5 mm). This suggests that the bacteria in question is more susceptible to non-polar chemicals, which have a higher affinity for extraction by n-hexane. This result emphasizes the intricacy of microbial resistance and the need for a wide variety of solvents in antimicrobial research (Osungunna, 2021).

The extracts showed different levels of efficacy in terms of antioxidant activity, as determined by the ABTS and DPPH tests. According to the ABTS test, the ethanol extract exhibited the maximum level of inhibitory activity at 9.73%, while the n-hexane extract showed the lowest level at 5.64%. According to the DPPH test, the methanol extract exhibited the maximum level of residual radical activity (99.68%), while the n-hexane extract had the lowest level (97.19%). The findings emphasize that various assays might provide distinct perspectives on antioxidant capabilities, and suggest that methanol may be more efficient in extracting molecules with potent abilities to scavenge free radicals (Sadowska-Bartosz & Bartosz, 2022).

It is concluded that ethanolic leaves' extract of *C. gigantea* had more phytochemical compounds as compared to others. Anti-bacterial and anti-oxidant activities showed promising results. Ethanolic extract revealed significant outcome against gram-negative bacteria. Methanolic and n-hexane leaf extract divulged convincing antibacterial activities against *Bacillus subtilis* and *Bacillus muralis* respectively. DPPH and ABTS assays demonstrated that the antioxidant activity of ethanolic and methanolic leaves extract is prominent. It can be employed in green synthesis in the future to boost medical and pharmaceutical applications.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hazqail Umar Khan: Main Concept, Writing -original draft, Investigation, **Muhammad Shahid Cholistani**: Resources, Visualization, Software, Formal Analysis, **Eliza Iqbal**: Visualization, Software, Formal Analysis, **Kashif Kareem**: Validation, Writing draft, Formatting, Grammar and Structure. **Hafiz Muhammad Kashif Zahoor**: Methodology, Formal Analysis, **Muhammad Farhan**: Investigation and Visualization, **Hafiz Shozab Ahmad Khan**: Visualization, **Muhammad Pervaiz Bhatti**: Supervision, and Validation. **Jallat Khan**: Supervision, Formal Analysis

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