UNVEILING THE NUTRACEUTICAL AND PHARMACOLOGICAL POTENTIAL OF RUBIA CORDIFOLIA L.: A PROXIMATE AND PHYTOCHEMICAL PERSPECTIVE

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Abstract

Introduction

Rubia cordifolia L., also known as Indian madder, is a medicinal plant renowned for its traditional uses. This study investigates the proximate and phytochemical composition, antioxidant, cytotoxic, and antibacterial properties of *ethanolic extracts from the roots, stems, and leaves of Rubia cordifolia* L. Through comprehensive analysis, key compounds, including alkaloids, flavonoids, and phenolics, were identified.

Methods

The proximate analysis procedure for *Rubia cordifolia* L. followed established standard principles (AOAC, 2005). phytochemicals were analyzed both qualitatively and quantitatively. The antioxidant activity was assessed using the DPPH radical scavenging assay. The DPPH assay demonstrated significant radical scavenging activity, with root extracts showing the highest inhibition percentage, comparable to that of the stem, leaves, and standard ascorbic acid. Cytotoxic effects were evaluated through Brine shrimp lethality assays, while antibacterial efficacy was determined against selected Gram-positive and Gram-negative bacterial strains using the well diffusion method.

Results

The proximate analysis revealed *Rubia cordifolia* L. to be rich in carbohydrates, fiber, and protein, with roots containing the highest amount of carbohydrates among all the plant parts. The phytochemical analysis of root, stem, and leaves determined secondary metabolites, including carbohydrates, proteins, alkaloids, phenols, flavonoids, fats, sterols, coumarins, glycosides, anthraquinones, and tannins, were present in the ethanolic extracts of the *R. cordifolia* L. plant sample. The quantitative analysis for phenols and flavonoids revealed the root extract to contain the maximum amount of flavonoids, whose values deviated from the reported literature. The DPPH assay showed significant radical scavenging activity, with root extracts demonstrating the highest inhibition percentage that is IC_{50}=2.57, comparable to stem, leaves, and standard ascorbic acid. Cytotoxic activity was calculated in the activity index and its LC_{50} values. Roots showed the maximum activity index as it has the lowest LC_{50} value. The antimicrobial activity of root, stem, and leaf extracts was assessed against gram-positive bacteria *(B. subtilis* and *S. aureus*) and gram-negative bacteria (*E. coli* and *P. aeruginosa*), with all the extracts inhibiting the growth of selected microorganisms on the agar plate. Roots have the highest antimicrobial inhibition effect with the lowest IC_{50} value of 830.92 µg/ml, followed by leaves and stems.

Conclusion

The results demonstrated significant bioactivity, indicating the therapeutic potential of *Rubia cordifolia* L. for pharmaceutical and nutraceutical applications.

Keywords: Proximate analysis, phytochemicals, antioxidant activity, cytotoxicity, antibacterial potential, natural therapeutics

Abbreviations:

- AOAC – Association of Official Analytical Chemists
- ATCC – American Type Culture Collection
- BSLA – Brine Shrimp Lethality Assay

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- DPPH – 2,2-Diphenyl-1-picrylhydrazyl
- GAE – Gallic Acid Equivalents
- LC_{50} – Lethal Concentration 50%
- WHO- World Health Organization, which

1. Introduction

Plants are rich in a variety of phytochemicals that have been used as a source of medicine for a very long time. Notably, the World Health Organization (WHO) reports that about 80% of the global population depends on botanical remedies for their primary healthcare Ekor, M., 2014). Over 6,000 plant species have been identified in northern Pakistan, many of which are used medicinally. Most of these plants are found in the region's hilly areas. Ahmed et al. (2019). Among these plants, *Rubia cordifolia* L. is used by locals in Gilgit to treat various ailments and also by locals in Poonch valley, Kashmir. Abbas et al. (2021).

The well-known ayurvedic herb *Rubia cordifolia* L., sometimes recognized as Indian madder or else manjishtha, is a flowering plant in the Rubiaceae family of coffees. It is a blooming plant that is only found in mountainous locations up to 3750 meters, primarily in the foothills of the Himalaya in Pakistan and India. In the Ayurvedic medical system, the plant is devoted to curing various illnesses, including skin disorders, blood purification, immunomodulation, anti-inflammatory, etc. In Eastern Asia and the Indian subcontinent, *Rubia cordifolia* L. is used as common traditional medicine. Chandraker et al. (2022). Table 1 shows the taxonomical classification of *Rubia cordifolia* L. Fig. 1 & 2 show the plant parts, i-e, roots, stem, and leaves of *Rubia cordifolia* L. Studies have found that *R. cordifolia* is rich in more than 100 compounds, mainly including anthraquinones, naphthoquinones, anthraquinone glycosides, naphthoquinone glycosides, bicyclic hexapeptides, triterpenoids and polysaccharides. A number of researches have reported that *R. cordifolia* has numerous pharmacological activities, including anti-inflammatory, anti-cancer, anti-tumor, antioxidation, antibacterial, anti-platelet aggregation, anti-nephrotoxicity, anti-urolithiasis, hepatoprotective effects, and neuroprotective effects. Wen et al., (2022).

Many studies have revealed that the anthraquinones found in the root extracts of *R. cordifolia* L. exert antimicrobial effects against a variety of bacteria, both gram-positive and gram-negative bacteria Chauhan et al., 2021; Qiao et al., 1990. Moreover, Basu and his group in 2005 revealed the methanolic extract to have a positive effect in inhibiting the growth of gram-positive as well as gram-negative bacteria, which were also inhibited by streptomycin and Penicillin G (standard) effectively. Wang et al. (1992). Researchers have examined the antimicrobial potential of *R. cordifolia* L. root extracts against different harmful bacteria. The presence of anthraquinones and flavonoids in these extracts proved effective in inhibiting the activity of *Gossypium phytopathogens.* Naidu et al. (2009). They found *Rubia cordifolia* L. to be rich in antimicrobial bioactive compounds. The compounds were isolated via column chromatography and identified using HPLC, GC/MS spectrometry. Out of the 55 plants studied, the methanolic root extracts of *Rubia cordifolia* L. produced the largest zone of inhibition and thus had the most potent antimicrobial activity.

Studies have revealed *R. cordifolia L. to* have antifungal properties. Methanolic and ethanolic root extracts of *R. cordifolia* L. have shown antifungal properties against *C. albicans.* Ismail et al. (2016). The antifungal activity of *R. cordifolia* L. has also been demonstrated by inhibitory action against 3 other fungi, *F. oxyporum, T. Phaseolina, and T. basicola* Okhti et al., 2020).

Mollugin extracted from the roots of *R. cordifolia* L. has been used to inhibit the expression of surface antigen of Hepatitis B virus. Ho et al. (1996). The aerial parts of *R. cordifolia* L. have also been seen to reduce viral replication. Sun et al. (2016).

The hydroxyl group found in the benzenes and anthraquinones in roots of *R. cordifolia* L. provides it with radical scavenging activity and allows it to behave as an antioxidant Cai et al., 2004). The methanolic and chloroform extracts have been reported to exert a

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gastroprotective effect by lowering several enzymes involved in oxidation, such as catalase and lipid peroxidase. Deoda et al. (2011). The ethanolic root extract of *R. cordifolia* L. has also been known to exhibit antioxidant properties by lowering the level of several enzymes Lodi et al., (2011). The present research, also aims to build a quantitative and data-oriented approach by analyzing proximate composition, phytochemical contents, and bioactivity profiles. Just as IoT-AI models optimize environmental data for accurate prediction, similar optimization principles can be applied in the nutraceutical field to improve phytochemical quantification, extraction efficiency, and bioactivity prediction. Mohandas et al., (2025)

2. Materials and Methods

2.1 Plant Collection and Extraction

The sample (*Rubia cordifolia* L.) was collected from Pahot, North of Pakistan (Karakoram Ranges, Gilgit Baltistan) based on ethnobotanical aspects. The herbaceous plant is native to hilly regions up to 3750 m, like the Himalayas, properly identified and authenticated by Dr. Rehmat Ullah Qureshi, Chairman, Department of Botany, and Dean, Faculty of Sciences, Arid Agriculture University, Rawalpindi. The plant materials were washed, air-dried at room temperature, and finely powdered. Ethanolic extraction was performed using the maceration technique for 72 hours with occasional stirring. The extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C. The dried extracts were stored at 4°C for further analysis.

2.2 Proximate Analysis

The proximate analysis of *R. cordifolia* L. involved assessing its carbohydrates, crude proteins, crude fibers, crude fat, ash content, and moisture content of the Root, stem, and leaves. The proximate analysis procedure for *Rubia cordifolia* L. was conducted following established standard principles (AOAC, 2005).

2.3 PHYTOCHEMICAL ANALYSIS

2.3.1 Qualitative Analysis of Phytochemicals

R. cordifolia L. was qualitatively assessed for phytochemicals such as alkaloids, flavonoids, terpenoids, saponins, phenols, coumarins, proteins, quinones, carbohydrates, and tannins was conducted using the standard procedure. Ugwu et al. (2020).

2.3.2 Quantitative Analysis of Phytochemicals

The quantitative tests for *R. cordifolia* L. were performed according to the standard procedures. Daniel et al. (2021).

The quantitative analysis was performed for the following:

1. Total phenol content
2. Total Flavonoid content

2.4 Antioxidant Activity Assays

The antioxidant activity of the extracts was determined using the following assay.

2.4.1 DPPH Radical Scavenging Assay

The scavenging ability of the extracts against DPPH free radicals was assessed at different concentrations, and the percentage inhibition was calculated. Ascorbic acid was used as a standard or positive control.

The antioxidant activity of the Plant separates against DPPH was assessed. Chaves et al. (2017). It was created to dilute DPPH to 0.0004 M in methanol. Each sample was extracted with 1 mL of methanol at various concentrations (50 g / mL, 100 g mL, and 150 g / mL), then 2 milliliters of methanolic DPPH dilution was added. Kept the tubes holding the combination at room temperature and in the shade for 20 minutes before determining the absorbance at 517 nm. The DPPH was

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diluted in methanol to create the blank or negative control. The resulting formulation was used to analyze the percentage of antioxidant scavenging activity for the extracts:

$$\text{Scavenging } \% = \frac{\text{CA}-\text{SA}}{\text{SA}} \times 100$$

Where, CA=Control absorbance SA= Sample absorbance

2.5 Cytotoxicity Assay

2.5.1 Brine shrimp lethality assay (BSLA)

In this assay, Artemia salina shrimp were consumed. Seawater was replicated, and its offspring were produced by Nauplii. Hamidi et al. (2014).

2.6 Antibacterial Activity

The antimicrobial activity of root, stem, and leaf extracts was assessed against the strains previously reported in the literature. Basu et al. (2005). Four different strains were selected for this study based on a systematic review of the literature. Two-gram positive strains *Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis* (ATCC 6051a), and two-gram negative bacteria *Escherichia coli* (25992) and *Pseudomonas aeruginosa* (ATCC 27853).

2.6.1 Reference Drug

Penicillin (1 µg and 10 µg), Kanamycin (30 µg), and Neomycin (10 µg) (Oxoid, UK) were used as reference drugs or as positive control. Ethanol was used as a negative control. Both antibiotics are broad-spectrum bactericidal antibiotics and tend to act on a large range of bacteria. Patel et al. (2021).

3. Results and Discussion

3.1 Proximate analysis

By AOAC (2005) guidelines, a proximate analysis of the roots, stems, and leaves of *Rubia cordifolia* L. was performed to ascertain the content of moisture, ash, crude protein, crude fiber, fat, and carbohydrates.

The moisture content was higher than previously reported, with leaves having the highest percentage (60.5%), followed by stems (44.5%) and roots (35.1%). Roots had the highest ash content (13.47%), followed by leaves (8.99%) and stems (7.06%), suggesting that inorganic metabolites were more prevalent. While fat content was highest in stems (7.13%), followed by leaves (6.94%) and roots (3.97%), crude fiber was most prevalent in stems (18.55%), followed by roots (12.01%) and leaves (8.5%). With possible pharmacological advantages, leaves had the highest crude protein levels (11.31%), followed by stems (8.77%) and roots (7.77%). Roots had the highest concentration of carbohydrates (27.58%), followed by stems (13.77%) and leaves (5.04%), which may indicate that they have antidiabetic effects. These results suggest that *R. cordifolia* L. might have substantial therapeutic and nutritional value.

The above Table 2 gives a summary of the proximate analysis of carbohydrates, proteins, fats, fibre, ash, and moisture content present in *R. cordifolia* L.

3.2 Phytochemical analysis of *Rubia Cordifolia* L.

The phytochemical analysis was performed in this study on the ethanolic extract of *Rubia cordifolia* L. to detect the presence of different metabolites both qualitatively and quantitatively. The results of different phytochemical tests are given below:

3.2.1 Qualitative Analysis of Phytochemicals

Qualitative analysis used rapid reagent tests that indicate the presence of a compound in a sample by color change in a reaction.

3.2.1.1 Qualitative tests for carbohydrates

Carbohydrates present in the plant extract were qualitatively confirmed by the Molish test, Fehling's test, and Benedict's test. Benedict's test and Molisch test were strongly positive for all the ethanolic extracts, i-e, root, stem, and leaves. Fehling's test was strongly positive for stems and roots while mildly positive for leaves. These tests indicate the presence of carbohydrates in the ethanolic extracts of *Rubia cordifolia* L., as previously indicated by Humbare et al. (2022).

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3.2.1.2 Qualitative tests for proteins

Proteins present in the extract were confirmed by standard qualitative tests for proteins (Xanthroprotic test, Biuret's test, Ninhydrin test, and Millon's test). The xanthoproteic test was strongly positive for all three extracts. Biuret's test was also positive for all three extracts. Millon's and ninhydrin tests were strongly positive for roots and stems but negative for leaves. These results were different from previously published literature by in which Millon's test and Ninhydrin test were negative for *R. cordifolia* L. root extracts. Chandrashekar et al. (2018).

3.2.1.3 Qualitative tests for alkaloids

Alkaloids present in the extract were confirmed qualitatively by standard tests (Hager's test, Mayer's test, and Wagner's test). Wagner's test, as well as Hager's test, was positive for all three extracts, while Mayer's test was only positive for leaves. These results are opposite to those reported by Sharma et al. (2013), where root, stem, and leaf extracts did not test positively for alkaloids. But variations do exist, as Humbare et al. (2022) reported alkaloids in root extract and Gupta et al. (2017) reported positive alkaloids in stem and leaf extracts.

3.2.1.4 Qualitative tests for phenols

Phenols in the sample were confirmed by the ferric chloride test (20 % $FeCl_3$) solution. The test was positive for roots and leaves but negative for stems. The presence of phenolics in plant extracts has also previously been reported in studies. Gupta et al. (2017).

3.2.1.5 Qualitative tests for flavonoids

Flavonoids were confirmed by the ferric chloride (10 % $FeCl_3$) test and the ammonia test. The tests were positive for all three: root, stem, and leaf extracts. The presence of flavonoids has also been reported previously by Sharma et al. (2013).

3.2.1.6 Qualitative tests for tannins

Tannins present in the sample were determined qualitatively by the bromine water test. The test gave strongly positive results for all three extracts, indicating that these extracts are rich in tannins. In other studies, *Rubia cordifolia* L. was reported to be poor in tannins Chandrashekar et al., 2018).

But Sharma et al. (2013) reported positive results for tannins in the alcoholic plant extract of *R. cordifolia* L.

3.2.1.7 Qualitative tests for saponins

A foam test was used to detect the presence of saponins. The foam test was positive for all three samples, indicating the presence of saponins in these samples. The results of this study contradict the results reported by Humbare et al. (2022) but are like those in Chandrashekar et al. (2018).

4.2.1.8 Qualitative tests for sterols

Salkowski test confirmed the presence of sterols in the sample. The test was positive for roots but negative for stems and leaves. The study reported sterols to be present in roots and leaves but absent in stems Humbare et al., 2022), while studies by Sharma et al. (2013) report it to be absent in all three extracts.

3.2.1.9 Qualitative tests for lipids

Stain test confirmed the presence of lipids in the sample. The results proved that roots contain the highest amount of oils, followed by leaves and then stems, as previously demonstrated by Humbare et al. (2022).

3.2.1.10 Qualitative tests for coumarins

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The presence of coumarins was confirmed by an alkaline sodium hydroxide test. Roots gave negative results for coumarins, while stems and leaves had positive results for coumarins. The presence of coumarins in the sample may vary as per geographical conditions, as the study proposed by Sharma et al. (2013) showed negative results for coumarins in roots, while that of Humbare et al. (2022) provided positive results for coumarins.

3.2.1.11 Qualitative tests for glycosides

The presence of glycosides was confirmed by Keller Keller-Kiliani test, which was positive for roots and negative for stem and leaf extracts. The results of this study are similar to those reported by Gupta (2017 and Chandrashekar (2018).

3.2.1.12 Qualitative tests for quinones

The ammonia test and the dilute hydrochloric acid test were both used to detect the presence of anthraquinones. The tests were positive for roots but gave negative results in the stem and leaves. These results are about the ones previously reported by Gupta et al. (2017) and Chandrashekar et al. (2018), whose studies also showed that roots were positive for anthraquinones and absent in stems and leaves.

The Phytochemical analysis of metabolites in this study varied somewhat from the ones reported earlier. These variations may be due to the extraction conditions and the environment in which the plant is grown. Different samples of plants obtained from different regions vary in their metabolite concentration due to the variable composition of minerals and nutrients in the soil Pant et al., 2021). The summary of the phytochemical analysis is shown in Table 3.

3.2.2 Quantitative Analysis of Phytochemicals

Quantitative analysis was performed in this study for the presence of total phenolic and total flavonoids in the ethanolic extracts of root, stem, and leaves of *Rubia cordifolia* L. Gallic acid was used as a standard or positive control.

3.2.2.1 Total phenolic content

Rubia cordifolia L. is rich in phenolic compounds, which have been reported in many studies, allowing it to act as an antimicrobial agent. Humbare et al. (2022). The phenolic content was calculated in triplets with dilutions of 250 mg/mL,500 mg/mL,750 mg/mL, and 1000 mg/mL of ethanolic extract of root, stem, and leaves. The phenolic content obtained from these is indicated in Table 4.

The lowest amount of phenolic content was recorded in GAE equivalents in mg/g. 24.74 mg/g recorded in the leaves, and the highest phenolic content was recorded in roots as 191.3 mg/g. The phenolic content values obtained from this study are slightly higher than those previously reported in *Rubia cordifolia* L. (Barlow et al., 2016, and Humbare et al., 2022). T t-test (one-tailed) was used to calculate significance. The results of the t-test were used to determine the probability scores. The results indicate that all of the p-scores are less than 0.05, suggesting that the data is statistically significant.

The figure shows that overall roots possess the highest amount of phenolics, followed by leaves and then stems. The values recorded are higher than previously reported. These variations may be due to environmental factors and the nutrient composition of the soil in which the plant is grown, imparting it with a higher value of total phenolics than normal.

3.2.2.2 Total flavonoid content

Total flavonoid content was measured in Quercetin equivalents QE (mg/g). For these dilutions of 50 mg/mL, 100 mg/mL, 150 mg/mL, 200 mg/mL, and 200 mg/mL were used the total flavonoid values obtained from this study are given in Table 5 below:

The data in the above table shows that flavonoid content increases linearly with increasing

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concentration of the sample. These values are almost similar to the ones previously reported in the literature. Humbare et al. (2022). T t-test was used to calculate significance, which was then used to estimate the *p*-score. The results of the t-test indicated all of the values to be statistically significant, having a *p*-score of >0.05.

The figure shows that the highest amount of Flavonoids is present in roots, followed by leaves, and then the lowest concentration of flavonoids is present in the stem. The highest concentration of flavonoids was 165.31 mg/g present in the roots, and the lowest concentration of flavonoids was 20 mg/g present in the stem.

In this study, the trend in total phenolics and total flavonoids is root > leaves > stem. The values obtained in our study are higher than those reported earlier, which might be due to the environmental influence, with soil containing more phenolic metabolites and thus imparting them to the plant.

The study also differs from others in terms of the concentration (mg/ml) of the sample used. Thus, the higher concentration (mg/ml) of the sample may also contribute to higher values of phenolics and flavonoids, as seen by the trend in graphs that the phenolic and flavonoid linearly increase with an increase in concentration.

This study also proves that phenolics are present in higher amounts (mg/ml) in roots, which further adds to their pharmacological importance. The phenolic compounds isolated and extracted from *Rubia cordifolia* L. have been known to have medicinal importance owing to their anticancer, antifungal, antimicrobial, and antioxidant properties. Because of this high quantity of phenolics, *Rubia cordifolia* L. extracts are being employed in the food and textile industry to help protect from microbes and their harmful effects.

3.3 Antioxidant Activity

The ethanolic extracts exhibited a dose-dependent antioxidant effect. The DPPH assay showed significant radical scavenging activity, with root extracts demonstrating the highest inhibition percentage, comparable to standard ascorbic acid. *Rubia cordifolia* L. possesses constituents that can be effectively exploited in the food industry as hemopreventive agents. Kaur et al. (2008). DPPH scavenging activity was calculated in the activity index and its IC_{50} values. The DPPH assay showed significant radical scavenging activity, with root extracts demonstrating the lowest IC_{50} value thus, highest scavenging activity i-e IC_{50} 2.57 µg/ml, comparable to stem 3.78 µg/ml, leaves 3.16 µg/ml, and standard ascorbic acid 2.76 µg/ml.

3.4 Cytotoxicity Analysis

The brine shrimp lethality bioassay is a valuable means for the primary evaluation of harmfulness. The results revealed that the lethality of compounds increases with an increase in their concentration. Roots shows LC_{50} = 0.48 and leaves LC_{50}=0.52 more toxic than standard potassium dichromate (0.99). Stem shows weaker cytotoxic activity than standard which is potassium dichromate.

R. cordifolia L. root shows the least LC_{50} value, thus, most potentially cytotoxic. *Rubia cordifolia* displayed greater action in cytotoxicity, it might be stated with the prominent cytotoxic effect of *R. cordifolia* L., Onocha et al. (2011).

3.5 Antibacterial Efficacy

Rubia cordifolia L. is known to possess antimicrobial properties owing to its being rich in phenolic compounds. Luo et al. (2022). Our study also determined the antimicrobial activity of this plant, and so to explore its use as a potential source for future antibiotics.

This study was designed to evaluate the antimicrobial effect of root, leaf, and stem extract of *R. cordifolia* L. against four bacterial strains (two gram-positive and two gram-negative bacteria). Gram-positive bacteria to be tested against were *Bacillus subtilis* and St*aphylococcus,* while gram-negative bacteria were *E. coli* and *P. aeruginosa.* Penicillin 10 µg and Neomycin 10 µg were used as positive controls.

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The results of antimicrobial activity were measured as zones of inhibition in millimeters, and the activity index was computed by comparing with the reference drug. The study yielded positive results with roots, stems, and leaves, all of them having a positive antimicrobial inhibitory activity against four selected strains.

The results summarized in the figure indicate the zone of inhibition and activity index of leaf, root, and stem extracts of *Rubia cordifolia* L. Root extract exhibits the highest antimicrobial activity among the three extracts, followed by stem, and the least amount of antimicrobial activity is exhibited by leaves. Moreover, the study also determined that the highest amount of antimicrobial activity was observed against *B. subtilis,* and the least amount of antimicrobial activity was observed against *P. aeruginosa*. The studies in Rani et al. (2010) indicated that the leaf extract of *R. cordifolia* L. has very little antimicrobial activity. While the study by Barlow et al. (2016) found it to have no antimicrobial activity.
The difference in antimicrobial activity might be due to some specific phenolics or anthraquinones that vary or differ in plants grown in different geographical locations, as the soil may impact the metabolites present in the plant sample. Fig. 8 & 9 show the activity with a zone of inhibition.

The figure shows that penicillin forms very little or no zones with the selected strains, indicating the possibility that these strains might be penicillin-resistant. Several studies have reported these bacteria to develop resistance to commonly used antibiotics, specifically penicillin. Kazemnia et al. (2014), Mancuso et al. (20121). *Bacillus subtilis* is, however, reported to be highly sensitive to penicillin (Yassin & Ahmad, 2012), as it contains five penicillin-binding domains, and penicillin is often recommended for the treatment of *B. Subtilis* infections. Andrews et al. (2002).

This study illustrates the potential of *R. cordifolia* L. to replace modern antibiotics, as it is rich in phenolics and anthraquinones that bestow it with great antibiotic potential, as it was able to inhibit bacteria that were resistant to penicillin. This ability of *R. cordifolia* L. may provide a way to use naturally occurring plants as a source to treat antibacterial infections, as the bacteria are becoming more and more resistant to synthetic antibiotics.

4.5.1 Minimum inhibitory concentration for antibacterial activity

Minimum inhibitory concentration, also called IC_{50} value, is defined as the minimum amount of sample concentration required to inhibit 50% of the bacterial growth. The IC_{50} values for all three samples were recorded and are given in Table 6 below

Table 6 shows the inhibitory (%) activity of root, stem, and leaf extracts of *R. cordifolia* L., control sample showing 0 inhibition, while it can be seen that the inhibitory effect of the plant increased with the increase in concentration. Stem has the highest IC_{50} value of 966.29 µg/ml, followed by leaves, which have an IC_{50} value of 858.78 µg/ml, and roots have the lowest IC_{50} value of 830.92 µg/ml. This result shows that roots have the highest antimicrobial inhibition effect, followed by leaves and then stems.

4. Conclusion

Rubia cordifolia L. has been widely used by the locals to treat a variety of ailments, including wound healing, inflammation, and pyrexia. This study focuses on the significant antioxidant, cytotoxic, and antibacterial potential of *Rubia cordifolia* L. ethanolic extracts. This study was conducted to analyze the phytoconstituents in *Rubia cordifolia* L. and the antimicrobial potential of its phytoconstituents. The proximate analysis revealed *Rubia cordifolia* L. to be rich in carbohydrates, fibres, and proteins, with roots containing the maximum amount of carbohydrates of all the plant parts. The phytochemical analysis of root, stem, and leaves determined that secondary metabolites, including carbohydrates, proteins, alkaloids, phenols, flavonoids, fats, sterols, coumarins, glycosides, anthraquinones, and tannins, were present in the ethanolic extracts of *R. cordifolia* L. plant sample. The quantitative analysis for phenols and flavonoids revealed the root extract to contain the maximum amount of flavonoids, whose values deviated from the reported literatureWith an IC_{50} value of 3.16 µg/ml*, R. cordifolia* L. exhibited notable free radical

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scavenging activity in the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay, which is frequently used to assess the capacity of plant extracts to scavenge free radicals. The positive control, ascorbic acid, had an IC_{50} value greater than, suggesting that *R. cordifolia* L. roots scavenges free radicals more effectively than ascorbic acid. This implies that the plant might provide strong defense against oxidative stress, a factor in several chronic illnesses.

Additionally, its significant cytotoxic activity, particularly in the Brine Shrimp Lethality Assay**,** suggests that *Rubia cordifolia* L. may have therapeutic applications in cancer treatment. *Rubia cordifolia* L. may persuade apoptosis and oxidative stress in cancer cells, leading to cell death. The antimicrobial activity of root, stem, and leaf extract was assessed against gram-positive *(B. subtilis* and *S. aureus*) and gram-negative (*E. coli* and *P. aeruginosa*), with all the extracts inhibiting the growth of selected microorganisms on agar plates.It may upset bacterial cell membranes and prevent essential enzyme functions, triggering bacterial death The results of antimicrobial activity were different from the literature reported earlier, in which extracts of *R. cordifolia* L. had very little or no effect on the gram-negative bacteria. The study surpluses *in vivo* validation and comprehensive molecular mechanism exploration. The findings support its traditional use in herbal medicine and underscore its potential for pharmaceutical applications. The capable cytotoxic and antibacterial activities of *Rubia cordifolia* L. propose its potential for improvement into novel plant-based therapeutics. The work bridges break between antibiotic resistance distresses and the potential of natural plant-based alternatives. Future research should focus on isolating active compounds and elucidating their mechanisms of action through *in vivo* and clinical studies. using data-driven and optimization-based models to achieve better accuracy, reproducibility, and predictive insight, whether for smart environmental systems or natural-product pharmacology.

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Table 1: Taxonomical Classification of *Rubia cordifolia* L. Chauhan et al. (2021).

Kingdom	**Plantae**
Class	Dicotyledoneae
Subclass	Sympetalae
Order	Rubiales
Family	Rubiaceae
Genus	*Rubia*

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Species	*cordifolia*

Table 2: Proximate analysis of root, stem, and leaves of *R. cordifolia* L.

	Leaves	Stem	Root
Moisture %	60.5 ± 0.5	44.5 ± 0.5	35.1 ± 0.1
Ash %	8.99 ± 0.09	7.06 ± 0.11	13.47 ± 0.03
Crude Fibre %	8.5 ± 0.04	18.55 ± 0.05	12.01 ± 0.04
Fat %	6.94 ± 0.05	7.13 ± 0.15	3.97 ± 0.02
Crude Protein %	11.31 ± 0.06	8.77 ± 0.02	7.77 ± 0.11
Carbohydrates	5.04 ± 0.1	13.77 ± 0.03	27.58 ± 0.03

Table 3: Quantitative analysis of phytochemicals in stem, root, and leaves of *R. cordifolia* L.

Test Name	**Stem**	**Root**	**Leaves**
Test for Carbohydrates			
Benedict's Test	++	+++	+++
Fehling's Test	++	+++	+
Molisch Test	+++	+++	+++
Test for Proteins			
Xanthoproteic test	+++	+++	+++
Millon's Test	-	+++	-
Biuret's Test	+	+	+
Ninhydrin Test	++	+++	-
Test for Alkaloids			
Hager's Test	+++	+++	+++
Mayer's Test	-	-	++

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Wagner's Test	+++	+++	+++
Test for Phenols			
Ferric chloride test	+	++	-
Test for Flavonoids			
$FeCl_3$ test	++	+++	++
Ammonia test	++	+++	++
Alkaline Reagent test	++	+++	++
Test for Tannins			
$FeCl_3$ Test	++	+++	++
Bromine water	+++	+++	+++
Test for Saponins			
Foam test	+++	+++	+++
Test for Sterols			
Salkowski's test	-	++	-
Test for Lipids			
Oil test	+	+++	-
Test for Glycosides			
Keller Kiliani test	-	+	-
Test for Coumarins			
Alkaline NaOH	+	-	+
Test for Anthraquinones			
Di-Hydrochloric acid	-	++	-
Ammonia test	-	++	-

** Quantitative analysis of phytochemicals in stem, root, and leaves of *R. cordifolia* L. ('-' sign shows negative result, '+' sign shows slightly positive, '++' sign indicates positive, '+++' indicates strongly positive)

Table 4: Total phenolic content in GAE equivalent (mg/g)

Concentration(mg/mL)	Root	Stem	Leaves
250	$58.67^{a} \pm 0.93$	$24.74^{a} \pm 0.09$	$71.4^{a} \pm 0.22$

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500	75.67[a] ± 0.13	71.32[a a] ± 1.0	84.3[a] ± 2.79
750	178.13[a] ± 1.54	85.58[a] ± 0.36	125.7[a] ± 0.18
1000	191.3[a] ± 0.05	100.13[a] ± 1.0	134.04[a] ± 0.42

Values are taken in triplets with ± standard deviation, "a" is a superscript indicating a significant value.

Table 5: Total flavonoid content in QE equivalent (mg/g)

Concentration(mg/mL)	Root	Stem	Leaves
50	53.36[a] ± 0.11	20.05[a] ± 0.05	26.74[a] ± 0.09
100	63.22[a] ± 0.11	26.67[a] ± 0.02	29.83[a] ± 0.24
150	97.91[a] ± 0.08	33.39[a] ± 0.04	37.67[a] ± 0.09
200	123.34[a] ± 0.01	35.42[a] ± 0.39	63.43[a] ± 0.14
250	165.31[a] ± 0.01	45.4[a]± 0.06	93.44[a] ± 0.17

Values are taken in triplets with ± standard deviation; a superscript indicates a significant value.

Table 6: Percentage inhibition by serial dilution of plant root, stem, and leaf extract at 100, 250, 500, 750, 1000µg/ml.

	Percentage Inhibition				
Sample	**100µg/ml**	**250 µg/ ml**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**
Control	0	0	0	0	0
Root	1.08±0.4	4.38±0.1	12.45±0.3	36.4±0.1	78.33±0.7
Stem	0.04±0.1	2.25±0.1	9.87±0.2	22.2±0.3	54.33±0.9
Leaves	1.78±0.1	3.33±0.2	19.90±0.2	38.3±1.4	65.14±1.

Figure :1 Stem &Leaves of *Rubia cordifolia* L.

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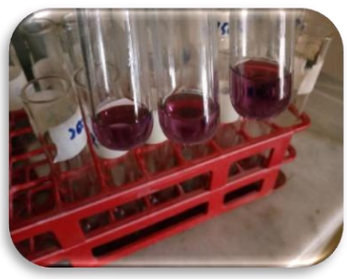
Figure :2 Roots of Rubia *cordifolia* L.

Figure 03: Shows the DPPH activity in which Change in the colure from violet to yellow was observed**:** a) Before Reaction b) After Reaction



a)

b)

Figure 04: Graph showing antioxidant activity of *Rubia cordifolia* L. using the DPPH assay

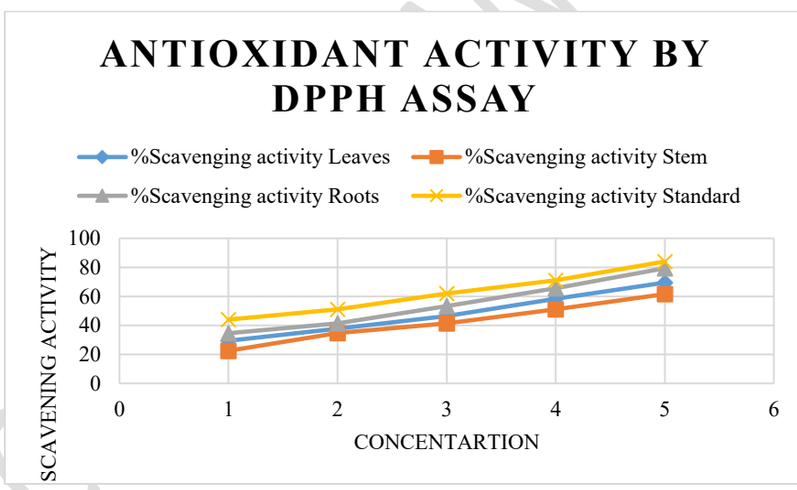


Figure 05: a) Shows setup for brine shrimp lethality assay b) Counting alive Nauplii

a)

b

Figure 06: Graph showing brine shrimp lethality assay of roots, stem, and leaves of *Rubia cordifolia* L. comparing standard or positive control Potassium dichromate.

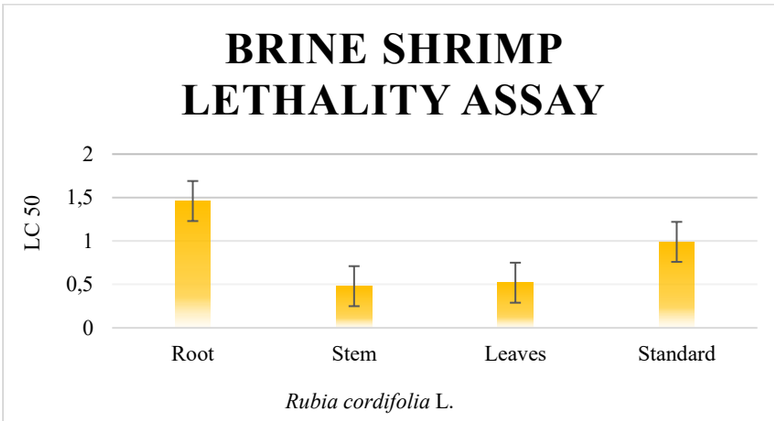


Figure 07: Graph showing antimicrobial activity of root, stem, and leaves of *Rubia cordifolia* L. against selected bacterial strains.

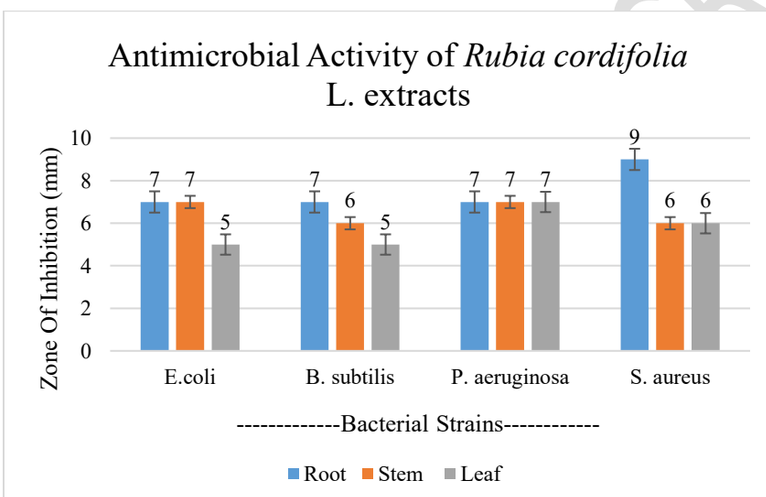


Figure 08: Comparative Zone of Inhibition of *Rubia cordifolia* L. extracts and neomycin (10 µg) against selected bacterial strains (positive control) and Ethanol as negative control.

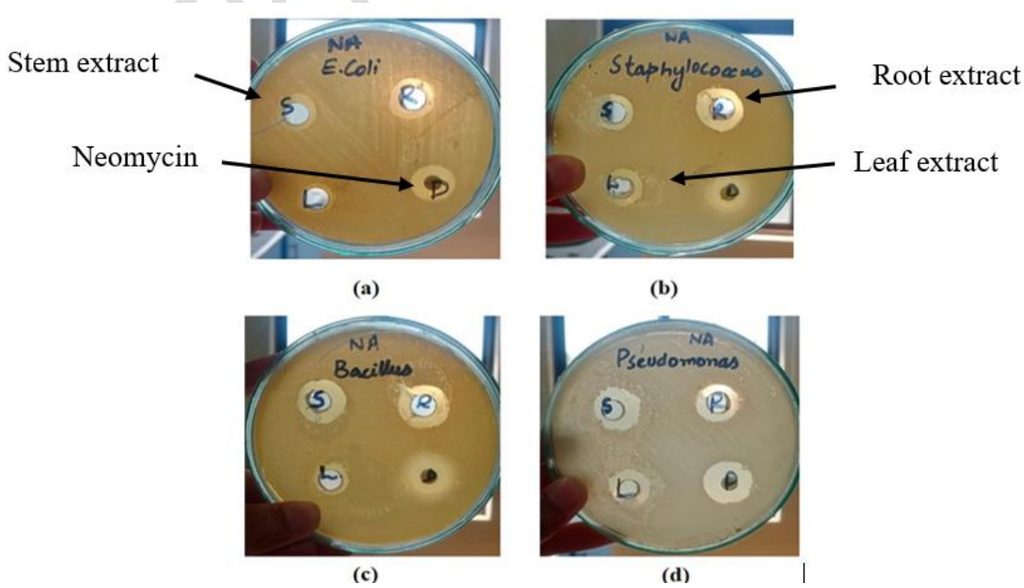


Figure 09: Comparative Zone of Inhibition of *Rubia cordifolia* L. extracts and penicillin (10µg) against selected bacterial strains (positive control) and Ethanol as negative control.

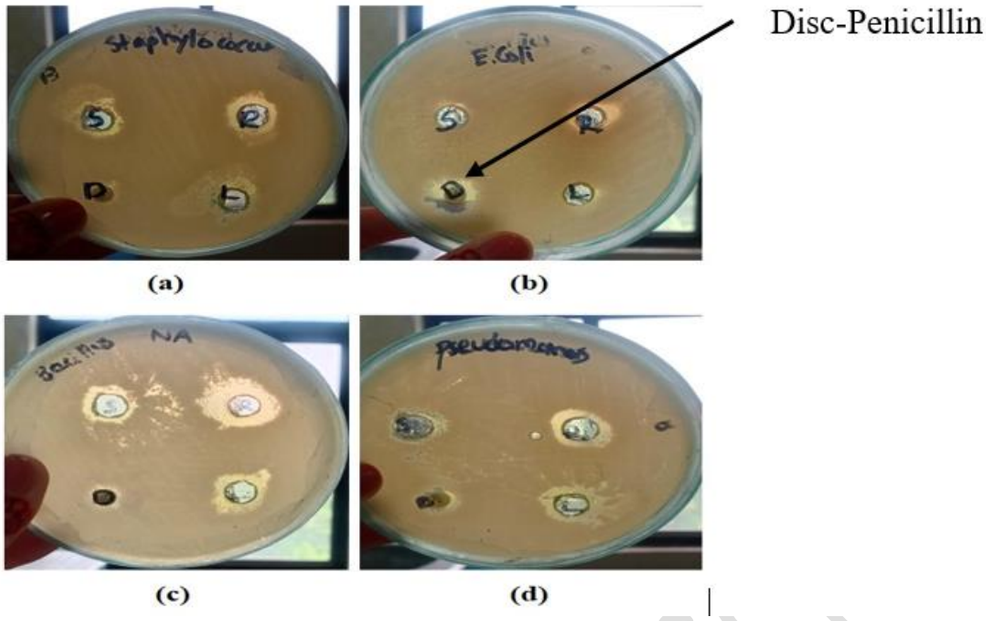


Figure :1 Stem &Leaves of *Rubia cordifolia* L.

Figure :2 Roots of Rubia *cordifolia* L.

Figure 03: Shows the DPPH activity in which Change in the colure from violet to yellow was observed**:** a) Before Reaction b) After Reaction.

Figure 04: Graph showing antioxidant activity of *Rubia cordifolia* L. using the DPPH assay

Figure 05: a) Shows setup for brine shrimp lethality assay b) Counting alive Nauplii

Figure 06: Graph showing brine shrimp lethality assay of roots, stem, and leaves of *Rubia cordifolia* L. comparing standard or positive control Potassium dichromate.

Figure 07: Graph showing antimicrobial activity of root, stem, and leaves of *Rubia cordifolia* L. against selected bacterial strains.

Figure 08: Comparative Zone of Inhibition of *Rubia cordifolia* L. extracts and neomycin (10 µg) against selected bacterial strains (positive control) and Ethanol as negative control.

Figure 09: Comparative Zone of Inhibition of *Rubia cordifolia* L. extracts and penicillin (10µg) against selected bacterial strains (positive control) and Ethanol as negative control.

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