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Original article

Antibacterial Activity, Phytochemical Screening, and Chemical Profiling of Leaves and Stems of the *Rubia* Plant

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Abstract
Plants as medicines have been used for thousands of years. Herbal extracts and formulations have long been regarded as a source of new and useful pharmaceuticals. The chemical composition of plant-based medicines has become a new interest these days. Several bioactive constituents of plants have been isolated and studied for various pharmacological studies. Rubia species are one of the earliest plant resources that possess commercial and important medicinal values. They were used as natural dyes in the old days and as drugs. The leaves and stems of Rubia plant were collected from Ain-Mara and Ras-Elhelal in the Al-Gabel Al –Kadar region during spring season of (2024) The results of phytochemical screening of aqueous and methanolic extracts of leaves and stems of Rubia plants showed absence of Anthraquinone in the two extracts, the Rubia leaves contain higher amount of total carbohydrates, amino acids, total protein, total phenols and antioxidant activity compared to Rubia stems, The anti-bacterial activity (mm) of aqueous extracts of Rubia leaves and stems did not appear any inhibition zones for E coli.
Keywords. *Antibacterial, Rubia Plant, Phytochemical, Stems, and Leaves.*

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Introduction

The plants are significant sources of medication used in all societies from ancient times. Different native plants are being used in the analysis, counteraction, and disposal of physical, mental, or social irregularities [1]. Restorative plants are the foundation of customary medicine and are vital to the well-being of people and networks. The restorative potential of these plants lies in certain synthetic substances that elicit distinct physiological effects on the human body [2].

Ayurveda pharmacopoeia has recorded more than 300 medicinal plants that are commonly used for medicinal purposes. The knowledge of chemical constituents present in plants helps scientists to understand the mechanism of drug action. It has been observed that the use of crude drugs obtained from different geographical regions showed large dissimilarity and variations in clinical results. The medications are gotten from the entiflowerst or from various organs, similar to leaves, stems, bark, roots, blossom, seeds, and so on. Some medications are made from excretory plant items, for example, gum, pitch, and latex. Phytochemistry is a part of science with phytochemicals, for example, synthetics acquired from the plant source. These mixes are known as auxiliary plant metabolites and have organic properties, for example, cancer prevention agent movement, antimicrobial impact, modulation of detoxification proteins, activation of the immune system, reduction of platelet aggregation, and adjustment of chemical digestion and anticancer properties [3]. Nutritional and proximate analyses of plants are used to evaluate their nutritional importance as they are being employed by humans for medicinal purposes [4]. Nature has endowed humanity with a wide variety of plants for exploration as food and medicine. The activity of these plants when consumed as food or taken as a drug is dependent on the chemical composition of their parts [5].

Several extracts, purified fractions, and components were found to exhibit antioxidant, antimicrobial, and anti-inflammatory activity [6] with little or no toxic effects. These include flavonoids, phenolic acids, glycosides, and tannins (Santos et al., 2016). These molecules constitute a principal group of secondary metabolites synthesized in plants that provide color and taste to most fruits and vegetables. Even though phenols are considered non-nutritive components, attention is being received due to their several positive effects [7]. Therefore, natural compounds are becoming more prominent in the marketplace since they are considered safe and non-toxic compared to synthetic antioxidants, which have limited use due to their various side effects [8]. In Libya, the environmental and chemical

Razi Med J. 2026;2(1):127-136
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https://razi.edu.ly/rmj/index.php/hm

studies were taken place in many studies on plants, water, air, and marine samples by using different instruments to evaluate the chemical and biological effects [9- 99]. This study was aimed to study the chemical contents, such as amino acids, total carbohydrate, anti-oxidant, and total phenols, beside investigate the extracts of leaves and stems of the Rubia plant growing in some Libyan regions.

Methods

In this study, the Rubia plant was collected from Ain-Mara and Ras-El-Helal in the Al-Gabel Al –Kadar region during the spring season of 2024. The collected samples were identified in Seliphium herbarium, Botany Department, Faculty of Science, Omar Al- Mukhtar University. The plant taxonomy was given in (Table 1).

Table 1. The taxonomy of the Rubia plant

Kingdom	**Plant Lavender**
Family	Rubioideae
Genus	Rubia L
Species	Rubia tinctorum L.

The leaves and stems of the Rubia plant were separated and washed several times with distilled water. The samples were then dried in a dark and dry place for two days at 37°C. Then the samples were ground by mortar and stored in polyethylene bottles until analysis.

Extract preparation

The Extraction was carried out according to the method described by previous studies [100]. Briefly, 10 grams of leaves and stems of the Rubia plant were weighed and mixed with 100 ml of two different solvents (distilled water and methanol) in a conical flask and kept in a rotatory shaker at 150 rpm for 4 hours. Then, the extract was evaporated under reduced pressure using a rotary evaporator apparatus and allowed to dry in the incubator till complete dryness.

Phytochemical screening

The following tests were performed to detect various phytochemical constituents that may be present in the studied plant extracts. All the phytochemical screening tests were carried out according to standard methods [9-16]. The methods are described as follows:

Screening for Carbohydrate Test

To 1ml of extract, 1ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes solution appeared green, showing the presence of reducing sugar.

Screening for Glycosides (Keller Kilianin Test)

5ml of each extract was added with 2 ml of glacial acetic acid, followed by the addition of a few drops of ferric chloride solution and 1ml of concentrated Sulphuric acid. The formation of the brown ring at the interface confirms the presence of glycosides.

Screening for Flavonoids: (Alkaline Reagent Test)

2 ml of the extracts were treated with a few drops of 20% sodium hydroxide solution, forming an intense yellow color, which becomes colorless on the addition of dilute hydrochloric acid, indicating the presence of flavonoids.

Screening for Anthraquinones

Bornträger's test

One ml of each extract of the successive aqueous ammonia or caustic soda extracts is added and shaken. Rose-red color in the aqueous layer develops in the presence of anthraquinone glycosides.

Razi Med J. 2026;2(1):127-136
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Modified Bornträger's test

One ml of each extract of the successive extracts of the tested herbal preparations is hydrolyzed with alcoholic potassium hydroxide, the acidified and continues as Bornträger's test. Rose-Red develops in the aqueous layer in cases of the presence of anthraquinones.

Screening for Saponins (Foam Test)

2ml of the extract was taken in a test tube, and 6ml of distilled water was added to it. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Determination of Carbohydrate

It was carried out as follows: One gram of each powdered sample was defatted with petroleum ether, then extracted with hot 80% ethanol twice. The combined extracts were evaporated till dryness, and the dried residues were dissolved in 10 ml of 10 % aqueous isopropanol in a volumetric flask. One ml of sample containing the equivalent of 20-100 µg glucose was pipetted into thick-walled test tubes of 16 mm-20 mm diameter. A reagent blank containing 1ml of water and a set of glucose standards (e.g., 25, 50 and 75 µg glucose, in a volume of 1 ml) were prepared at the same time. One ml of 5.0% (W/ V) phenol was added to all tubes and mixed. Then, from a fast-flowing stream, 5 ml of concentrated sulphuric acid was added, directing the stream of acid on the surface of the liquid and shaking the tube simultaneously, to effect fast and complete mixing. The tubes were allowed to stand 10 min, shaken, and placed in a water bath at 25°C to 30°C for 20 min. before readings were taken. The colour was stable for several hours. The absorbance of the characteristic yellow colour was measured at 490 nm, followed by Beer's law of a standard calibration curve of glucose [101].

Estimation of total soluble protein: Crude protein was determined by converting total nitrogen to total protein (Total N × 6.25), and protein was expressed as mg protein/g.

Estimation of amino acids

A dry defatted sample of 0.1 g was hydrolyzed with 10 ml of 6 N HCl in sealed tubes for 24 hrs at 110°C. After hydrolysis, the excess HCL was removed by evaporation under vacuum with occasional addition of water. The residue was dissolved in sodium citrate buffer, pH 2.2, and any insoluble matter was filtered off. The optically clear solution (30 µl) is chromatographed in an amino acid analyzer, at the unit of analysis and scientific services, faculty of agriculture, Alexandria University.

Determination of total phenols by Folin Ciocalteu Method

The phenolic content was quantified using the Folin-Ciocalteu method, with gallic acid as the standard. Briefly, 10 mL of each extract was mixed with 3 mL of distilled water and Folin-Ciocalteu reagent. Samples (barley leaves and seeds) were placed in test cuvettes, followed by the addition of 0.5 mL Folin-Ciocalteu reagent and 2 mL of 20% sodium carbonate (Na_2CO_3). After incubation for 1 minute and cooling for 15 minutes, the absorbance was measured at 650 nm using a UV-Vis spectrophotometer. The results were calculated as milligrams of gallic acid equivalent per gram of fresh weight [102].

Determination of antioxidant activity by the Prussian blue method

One gram of the powdered sample was defatted with petroleum ether. The defatted powder was then extracted sequentially by stirring with 10 ml of methanol twice, then extracted again with 10 ml 1% hydrochloric acid: methanol (v/v). The three combined extracts were evaporated under vacuum, and the residue was dissolved in 10 ml methanol. Half a milliliter of the solution was diluted with 3 ml of distilled water, 3 ml (0.008 M) of $K_3Fe(CN)_6$ was added,3 ml of 0.1M HCl, and 1 ml 1% $FeCl_3$. The blue color is allowed to develop for 5 minutes, and the absorbance is measured at 720 nm in the central lab of the Faculty of Science, Omar Al-Mukhtar University [103].

Antibacterial activity

The agar well diffusion method was followed to determine the antibacterial activity. Mueller-Hinton agar (MH), Plates were swabbed (sterile cotton swabs) with pathogenic bacteria, wells 4 mm in diameter were made in each of these plates using a sterile cork borer, and about 100 µl of different organic solvents. Were added by sterile syringe

Razi Med J. 2026;2(1):127-136
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Razi Medical Journal
https://razi.edu.ly/rmj/index.php/hm

into wells. The plates were incubated at37 °C for 18 – 24 h. The zone of inhibition was measured using a meter rule as described by previous studies [104].

Results

Table 2 showed the phytochemical screening of aqueous extracts of leaves and stems of Rubia plants. The results revealed that alkaloid only found in high amounts in the Rubia stems, while anthraquinones were absent in the leaves and stems of Rubia plants. The high content was only present for alkaloids in Rubia stems.

Table 2. The phytochemical screening of aqueous extracts of the leaves and stems of the Rubia plant.

Phytochemical screening test	Parts of the Rubia plant	
	Rubia leaves	Rubia stems
Carbohydrates and/or glycosides	+	+
Tannins	+	++
Alkaloid	++	+++
Flavonoids	++	+
Anthraquinone	-	-
Saponins	+	+

Table 3 showed the phytochemical screening of methanol extracts of leaves and stems of Rubia plants, which revealed that tannins were found in high amounts in leaves and stems of Rubia plants. While anthraquinones were absent in the leaves and stems of Rubia plants. Anthraquinones were absent in Rubia stems and leaves.

Table 3. The phytochemical screening of a methanolic extract of the leaves and stems of the Rubia plant.

Phytochemical screening test	Parts of the Rubia plant	
	Rubia leaves	Rubia stems
Carbohydrates and/or glycosides	+	+
Tannins	+++	+++
Alkaloid	+	+
Flavonoids	++	++
Anthraquinone	-	-
Saponins	++	++

Table 4 presented the total carbohydrate contents, total amino acids, and total protein contents (ppm) of the Rubia plant extracts. The results revealed that the content of all previously mentioned parameters in leaves was higher than the content in stems. This study stated that the high total phenol content and the high antioxidant capacity.

Table 4. The total carbohydrate contents, total amino acids, total proteins, and total phenol contents (ppm) of the leaves and stems of the Rubia plant extracts.

Content / Sample	Total carbohydrate	Amino acids	Total protein
Rubia leaves	0.840	0.625	1.223
Rubia Stems	0.442	0.259	0.473

Table 5 revealed that the content of phenolic compounds and antioxidant activity are directly related, as the higher the content of phenolic compounds in the leaves or stems of the Rubia, the higher its content of antioxidant activity.

Razi Med J. 2026;2(1):127-136
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Table 5. The total phenols and antioxidant activity contents (ppm) of the leaves and stems of the Rubia plant extracts.

Content / Sample	Total phenols	Antioxidant capacity
Rubia leaves	42.16	80.11
Rubia Stems	10.6	50.97

Table 6 presented the anti-bacterial activity (mm) of aqueous extracts of Rubia leaves and stems on the selective bacterial species. The results revealed that the aqueous extracts of Rubia leaves and stems did not affect the E coli bacteria, while Staphylococcus showed an effect in varying proportions.

Table 6. The anti-bacterial activity (mm) of aqueous extracts of Rubia leaves and stems on the selective bacterial species.

Type of bacteria	Rubia Leaves				Rubia Stems			
	100 %	75 %	50 %	25%	100 %	75 %	50%	25 %
Staphylococcus	3	6	0	0	5	5	3	0
E-coli	0	0	0	0	0	0	0	0

Table 7 showed the anti-bacterial activity (mm) of methanol extracts of Rubia leaves and stems on the selective bacterial species. The results revealed that the inhibition zones of all concentrations (100, 75, 50 and 25%) on Staphylococcus of Rubia leaves were higher than the inhibition zones of all concentrations (100, 75, 50 and 25%) on E coli of Rubia stems, The higher the concentration of either the leaf or stem extract of the Rubia plant, the greater the area of inhibition zones for both types of bacteria.

Table 7. The anti-bacterial activity (mm) of methanol extracts of Rubia leaves and stems on the selective bacterial species.

Type of bacteria	Rubia Leaves				Rubia Stems			
	100 %	75 %	50 %	25 %	100 %	75 %	50%	25 %
Staphylococcus	11	8	6	2	8	7	4	1
E-coli	6	5	5	4	7	7	6	5

Discussion

The difference in the components of the two parts is, according to some authors, related to several factors. Among them are the morpho-anatomical structure, the physiological activity of the different parts of the plant, geographical factors, climatic conditions, and gene expression [105]. It was reported thatthe ethanol extract of the R. cordifolia powder contains alkaloids, flavonoids, and terpenes, while the methanol extract showed the presence of alkaloids, flavonoids, glycosides, and terpenes. The aqueous extract of plant powder showed the presence of alkaloids, saponins, glycosides, and terpenes [106]. The bioactive compounds are of the primary metabolism of the species. Therefore, plants are regarded as a valuable repository of usual chemical compounds, since these bioactive compounds are usually chemical fingerprints of individual species. Approximately 80% of people in developing countries depend on plant-based traditional medicines for their primary health care needs [107].

A wide range of medicinal plant parts possessing a variety of pharmacological activities, such as root, stem, bark, leaves, flowers, and fruit extracts, are used as powerful raw drugs. The plant-derived drugs reflects its recognition of the validity of many traditional claims regarding the value of natural products in health care. Knowledge of the proximate composition of the raw material allows for determining its possible use in medicine, cosmetology, pharmacy, and the food industry. For pharmacy and medicine, research on determining the biological activity of the plant, mainly antioxidant activity, is of the greatest importance. The antioxidant effect protects tissues and organs against the harmful effects of free radicals, leading to the development of diseases (e.g., cancer, cardiovascular) [108]. Several extracts, purified fractions, and components were found to exhibit antioxidant, antimicrobial, and anti-inflammatory activity with little or no toxic effects. These include flavonoids, phenolic acids, glycosides, and tannins [109]. These molecules constitute a principal group of secondary metabolites synthesized in plants that provide color and taste to most fruits and vegetables. Even though phenols are considered non-nutritive components, attention is being paid due to their several positive effects. Therefore, natural compounds are becoming more prominent in the

marketplace since they are considered safe and non-toxic compared to synthetic antioxidants, which have limited use due to their various side effects [110].

Medical plants have started to be considered an essential source in treating/preventing various kinds of disease. Each plant consists of several important ingredients that can be used in the medical field and can be involved in the development of different kinds of drugs. Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purposes, including the treatment of infectious diseases. Currently, many studies warn people about the risks and dangers of pathogenic microorganisms that have become resistant to discovered antimicrobials [111]. Polyphenolic compounds are documented as good natural antioxidants that act as scavengers of radicals or metal chelating agents. Several extracts, purified fractions, and components were found to exhibit antioxidant, antimicrobial, and anti-inflammatory activity with little or no toxic effects. These include flavonoids, phenolic acids, glycosides, and tannins (Santos et al., 2016). These molecules constitute a principal group of secondary metabolites synthesized in plants that provide color and taste to most fruits and vegetables.

Even though phenols are considered non-nutritive components, attention is being received due to their several positive effects [112]. Therefore, natural compounds are becoming more prominent in the marketplace since they are considered safe and non-toxic compared to synthetic antioxidants, which have limited use due to their various side effects [113]. The use of plant extracts, some chemical complexes, and phytochemicals, with known antimicrobial properties, can be of great significance in treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds, which are part of the essential oils as well as in tannin [114-118].

Conclusion

Our study of the leaves and stems of the Rubia plant revealed a considerable number of phytochemicals in both the aqueous and methanolic extracts. This study also indicated the presence of small amounts of amino acids, carbohydrates, and proteins. In addition to total phenols and antioxidant activity, there was also limited activity against *E. coli* and *Staphylococcus* bacteria, whether the aqueous or methanolic extract, while the aqueous extract did not show any activity against E. coli bacteria. Therefore, it is recommended to conduct further studies on such medicinal plants, identify their various components, use multiple solvents, and determine their effectiveness against other types of pathogenic organisms.

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Conflict of interest. Nil

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