

Phytochemical Screening and Antibacterial Activity of Methanol Extracts of *Indigofera tinctoria*

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ABSTRACT

This study aims to identify the secondary metabolite content and evaluate the antibacterial potential of the methanol extract of *Indigofera tinctoria* leaves. The research was conducted using an exploratory laboratory approach, which involved several key stages sample preparation, ultrasound-assisted extraction with methanol, phytochemical screening, and antibacterial activity testing by the agar disk diffusion method. Phytochemical screening used specific reagents, such as iron (III) chloride, Wagner's, Dragendorff's, and Liebermann-Burchard reagents to detect secondary metabolites. The results of the phytochemical analysis showed that the analysis revealed that the methanol extract of *Indigofera tinctoria* contains various bioactive compounds, including flavonoids, alkaloids, and steroids. Antibacterial activity testing showed the extract inhibited the growth of both gram-positive and gram-negative bacteria. Notably, the extract exhibited the greatest effectiveness against gram-positive bacteria, with the highest inhibition zone recorded in *Bacillus subtilis* (19.45 mm), followed by *Staphylococcus aureus* (17.30 mm), *Escherichia coli* (15.55 mm) and the lowest in *Salmonella typhi* (8.80 mm). The results suggest that the methanol extract of *Indigofera tinctoria* leaves processes significant antibacterial activity, particularly against gram-positive bacteria, and highlight its potential as a source of natural antibacterial agents for future pharmaceutical applications. Further research, including in vivo studies and the isolation of individual compounds, is recommended to understand better the mechanisms involved and to assess the safety and efficacy of *Indigofera tinctoria* as a therapeutic agent.

KEYWORDS

Indigofera tinctoria, Phytochemical Screening, Flavonoid, Alkaloid, Steroid, Antibacterial Activity

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A. Introduction

Indonesia, a tropical country with high biodiversity, is home to the tarum plant (*Indigofera tinctoria*). Widely distributed throughout the country, this plant serves not only as commonly used natural dye but also as a source of important bioactive compounds. Any member of the genus that is an annual plant or shrub displaying an indigo color after flowering is generally referred to as an indigo plant. Most of the approximately 700 species in this group are found in tropical regions (DN et al., 2026).

Indigofera tinctoria has small, hairless, odd-pinnate leaves with five to eleven ovate leaflets. In contrast, closely related species have green stems and no red pigments. The plant displays large sepals, wings, and pink flower on upright, axillary racemes 4.56-15.25 cm long. The pods are cuboid or cylindrical, containing three to eleven oblong, cylindrical, dark green seeds. Leaf anatomy shows a distinct mesophyll and aligned palisade cells. The stem has a rectangular cross-section with

secondary xylem, phloem, and pith. Roots feature dense secondary xylem typical of an actinostele arrangement ([Shivani, 2025](#)).

Indigofera or tarum is a group of tiny wild bushes. Flavonoids, which are *Indigofera* glucoside chemical compounds found in the leaves, are naturally occurring pigments that, when hydrolyzed by enzymes and oxidized using oxygen rich air, can generate blue or indigo in extracts from *Indigofera* leaves ([Madina et al., 2024](#)).

The legume plant *Indigofera tinctoria* is native to tropical Asia and some regions of Africa. This plant is a member of genus *Indigofera*, species *Tinctoria*, family *Fabaceae*, subfamily *Faboideae*, and Kingdom *Plantae*. It is a shrubby herbaceous plant that grows to 1-2 meters tall. Its stems are upright and heavily branched. Its leaves are compounds, with the petiole measuring 2.5 to 10 centimeters. Its leaflets are 5 to 13 in number and oval, oblong, obovate in shape, 10 to 27 millimeters long, 4 to 17 millimeters broad; its flowers are pink, standard orbicular-obovate, 4.2 mm long; the pods are glabrescent, 15 to 15 (usually 6 to 12), much longer than broad. It grows well along riverbanks, roadsides, and grassy fields, and is frequently grown in tropical climates up to 300 meters above sea level. Plants may die from excessive watering or persistent rain. According to reports, the common indigo plant can withstand 6.4 – 41.0 dm of annual precipitation, a mean annual temperature of 10.5 – 40.4 °C, and a pH of 4.3 – 8.7 in the soil ([Safitri et al., 2025](#)).

Light has a significant impact on the indigo pigment synthesis. This is because the shikimic acid pathway produces nitrogen containing indigo precursors. Pentose phosphate and glycolysis are converted into aromatic amino acids via the shikimate pathway. Light intensity and nutrient availability have a major impact on plant development and production. The amount of nutrients required to maximize *Indigofera tinctoria* growth and yield changes with light intensity ([Budiastuti et al., 2021](#)). Light, as the key environmental factor, influences survival rate, morphology, photosynthetic traits, and primary and secondary metabolism in plants. Plants depend on light, and a lack of it can directly affect the development of their photosynthetic machinery. Furthermore, light availability significantly affects the majority of pigments associated with thylakoids and the photosynthetic system ([Setyaningrum et al., 2021](#)).

Low light intensities specifically enhance the production of secondary metabolites in *Indigofera tinctoria* plants. Studies show that at 25% and 10% light, *Indigofera* produces its highest levels of secondary metabolites. Therefore, cultivating *Indigofera* under low light conditions is necessary for high metabolite production ([Huda et al., 2022](#)).

True indigo, or *Indigofera tinctoria* is a commercially and medicinally significant plant that produces dyes. This plant's natural indigo dye yields a rich blue hue. The plant is known as “Blue Gold” for its substantial commercial value. This plant is indigenous to Asia and may be found in Africa, the Americas, South Asia, and Southeast Asia. It grows in Thailand, China, Pakistan, and other Eastern nations. It is grown in Tamil Nadu, Kerala, Andhra Pradesh, Uttarakhand, and other parts of India ([Thanuja; Sonia; Nair, 2026](#)).

One plant that has long been used in traditional medicine to cure infections is *Indigofera tinctoria*. This plant's positive reputation for possessing antibacterial qualities is supported by recent studies. Strong antibacterial properties were observed in methanol and ethanol extracted plant extract, particularly against a variety of clinically significant bacteria, including *Salmonella typhi*, *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus* (including its methicillin resistant strains). Its extensive phytochemical composition, including alkaloids, flavonoids, saponins, tannins, and cardiac

glycosides, is responsible for its antibacterial activity. The disk diffusion technique and minimum inhibitory concentration (MIC) tests, among other antimicrobial assays, demonstrate strong antibacterial activity, with inhibition zones ranging from 6 mm to 25 mm, depending on the type of extract used ([Shivani, 2025](#)).

To achieve the Sustainable Development Goals (SDGs) set by United Nations (UN), renewable energy technology is of paramount importance. In this context, natural dyes derived from *Indigofera tinctoria* (indigo) can serve as inexpensive, environmentally friendly photosensitizers for Dye Sensitized Solar Cells (DSSCs) ([Syafri et al., 2025](#)). Through nitrogen fixation, *Indigofera tinctoria* can enhance soil fertility and biodiversity while thriving in tropical climates and degraded soils with little input. By turning its biomass into compost or biochar, it can lessen the need for artificial fertilizers and increase resource efficiency. These methods align with Sustainable Development Goals (SDGs) 12, 13, and 15, which promote climate resilient agriculture, sustainable production, and ecosystem restoration ([Rao et al., 2025](#)).

The study of phytochemicals generated by plants, including their structural makeup, biosynthetic routes, roles, and mechanisms of action in living systems, is known as phytochemistry. Phytochemicals, compounds found in plants, are divided into primary and secondary metabolites based on their structures and metabolic pathways. The structural characteristics of primary metabolites are quite similar. Additionally, they are essential for plant growth, metabolism, development, and survival. Primary metabolites include things like proteins, lipids, carbohydrates, and nucleic acids. Although secondary metabolites are not essential to a plant's life, they do influence how the plant interacts with its environment and, consequently, its place in the ecosystem. Secondary metabolites include polyphenols, flavonoids, alkaloids, saponins, cardiac glycosides, and anthocyanins ([Thacker & Ram, 2024](#)).

Phytochemicals are unique to specific plants and plant components. They protect plants against harmful substances, such as insects, bacteria, and diseases, as well as conditions like high salinity and high temperatures. They are also responsible for giving the plant its color. Conventional plant derived medicines are favored over synthetic ones because they are less harmful to the environment and have fewer adverse effects. Phytochemicals are plant compounds with a wide range of bioactivities and several health benefits. They have also been used in traditional medicine to treat several illnesses and disorders. A wide spectrum of bioactivities, including antioxidant, anticancer, analgesic, antimicrobial, antidiarrheal, and more, are present in plants that contain different bioactive phytochemicals ([Thacker & Ram, 2024](#)).

To find bioactive chemicals, a phytochemical analysis *Indigofera tinctoria* was carried out utilizing a number of qualitative and quantitative techniques. Fresh leaves were air dried, ground, and extracted with secondary solvents, including petroleum ether, chloroform, ethyl acetate, methanol, and ethanol, either independently or in different combinations, for qualitative analysis. Phytochemical screening has revealed the presence of almost all of them in the early phases, including alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides, phenolics, carbohydrates, steroids, proteins, and amino acids ([Shivani, 2025](#)).

Finding the bioactive substances found in plants which are frequently a sign of a plant's potential therapeutic value begins with phytochemical screening. This method has been widely used for microalgae, traditional drinks, and a variety of medicinal plants parts, including leaves, flowers, seeds, fruits, and stems. Such widespread use demonstrates Indonesia rich biodiversity and the

increasing interest in using regional plants as sources for traditional remedies and phytopharmaceuticals. A variety of secondary metabolites, including flavonoids, tannins, saponins, steroids, triterpenoids, polyphenols, glycosides, and anthraquinones, are tested during phytochemical screening. Among the most often found substances in these investigations are flavonoids and tannins. Flavonoids have anti-inflammatory, anticancer, and antibacterial actions in addition to their well-known antioxidant qualities. The antiseptic, antibacterial, and astringent properties of tannins are well known, especially in relation to wound healing. Saponins and alkaloids, which provide further pharmacological advantages, including immune stimulating and analgesic effects, are also present in many plant samples. However, different plant species vary in the levels of these chemicals. For example, golka leaves lack flavonoids and saponins and solely contain alkaloids and steroids. On the other hand, almost all the chemicals studied are found in plants such as chickpea fruits, onion bulbs, and katuk leaves. These variations highlight how a plant's species, the portion of the plant examined, the environment, and the extraction techniques or solvents used all significantly impact phytochemical profiles ([Putri et al., 2024](#)).

One of secondary metabolites found in plants is flavonoids, which generally have a polar structure due to the presence of an OH group that creates hydrogen bonding. One of the secondary metabolites with antimicrobial, antifungal, antiviral, anticancer, and antitumor properties is flavonoids. Furthermore, flavonoids have antibacterial, antiallergic, cytotoxic, and antihypertensive properties. The roots, leaves, flowers, and fruit all contain flavonoids. All green plants contain flavonoids, secondary metabolites with a variety of pharmacological properties ([Putri et al., 2024](#)).

Flavonoids have a basic C6-C3-C6 structure. They form a broad class of naturally occurring polyphenolic compounds with two aromatic rings (A and B) joined by a single heterocyclic ring (C). Variations in oxidation, the amount and position of hydroxyl groups, and glycosylation patterns create subclasses with notable compounds and unique structural features. These differences in substitution and structure determine chemical characteristics, solubility, and how each compound interacts with biological targets. This structural diversity is key to understanding structure-activity relationships (SAR) in medicinal chemistry. The addition of prenyl or geranyl groups, for example, increases lipophilicity and strengthens interactions with target proteins and cell membranes, which influences the anticancer and anti-inflammatory potential ([Amin & Wulandari, 2025](#)).

Alkaloids, a broad class of natural compounds distinguished by nitrogenous bases, are commonly found in many plants, including medicinal species (Tanfil et al., 2023). As a major group of secondary metabolites, alkaloids exhibit diverse structures and a wide range of biological functions. Although most originate from plants, these nitrogen-containing compounds are key in synthesizing analogs with medical applications such as analgesics, antibacterial drugs, and antineoplastic agents. Understanding the structure and classification of alkaloids is crucial for linking their properties to biological activities and advancing drug discovery and development ([Mohammed et al., 2024](#)).

Alkaloids are a type of naturally occurring chemical compounds that are mostly composed of nitrogen atoms, which are frequently folded into a heterocyclic ring-like shape. These chemical components are widely distributed across the globe in plants, but they have also been found in some fungi, bacteria, and animal species. The phrase “alkali-like” first used by Carl F. W. Meissner in 1819 and referring to these compounds' fundamental (alkaline) characteristics, gave them the name “alkaloid”. Alkaloids have played a significant role in human medicine and society throughout history. Due to their potent physiological effects, several of these drugs were frequently used as stimulants

in ancient medicine and eventually led to the creation of contemporary medications. Morphine, caffeine, nicotine, and quinine are a few well known examples of alkaloids that are primarily used as medications or as drugs having psychotropic effects ([Talreja & Tiwari, 2024](#)).

Three cyclohexane and one cyclopentane ring make up the triterpenoid chemical steroid, which is present in the nucleus of cyclopentane perhydrofenanthrene. In addition to improving metabolism, sexual function, and other biological processes, steroids help lower the body's salt levels. It has been demonstrated that plant steroids can effectively reduce cholesterol and prevent cancer. Steroids and terpenoids, like those found in the stimulant isolate Temulawak (*Curcuma xanthorrhiza roxb*), are metabolites found in many drugs. Furthermore, steroids are present in *Curcuma xanthorrhiza roxb* and *Mahogany seed* extract. Terpenoids and steroids are also present in broken ground stem (*Euphorbia tirucalli L*) and natural plant extract *Phyllanthus niruri L*. ([Nola et al., 2021](#)).

Steroids, a key class of plant secondary metabolites, are especially valuable to the medical industry. Currently, over 150 steroids are registered as a drugs. For example, estrogen used as a contraceptive is a steroid sex hormone that inhibits ovulation. Progestin, a synthetic steroid, is used for pregnancy tests and to prevent miscarriage. Glucocorticoids, prescribed for allergies, fever. Leukemia, and hypertension, serve as anti-inflammatory drugs. Cardiac glycoside steroids like cardenolides are used as a heart tonics and diuretics. Meeting growing demand for these drugs will require better access to abundant steroid raw materials in the future ([Rezeki et al., 2023](#)).

Indigofera tinctoria demonstrated antioxidant hepatoprotective, anticancer, antiseizure, antidyslipidemic, and antibacterial properties. Specifically. *Indigofera tinctoria* leaf extract showed strong antioxidant activity, with an IC²¹ value of 51.66 µg/mL. Furthermore, *Indigofera tinctoria* antioxidant activity has been extensively studied utilizing various solvent extracts. *Tinctoria* of leaves demonstrated substantial antioxidant activity, including high DPPH and nitric oxide scavenging capacity and lipid peroxidation inhibition (87-96%). Additionally, it was discovered that the ethyl acetate, methanol, and aqueous extracts had significant antihemolytic and metal chelating properties in addition to having larger concentrations of phenolics and tannins. *Indigofera tinctori's* aqueous extract has high concentrations of flavonoids (75.43 ± 3.36 mg/g), total phenolics (267.2 ± 2.42 mg/g), and total antioxidants (349.11 ± 8.04 mg/g). significant free radical scavenging efficacy against DPPH (52.08%), nitric oxide (23.12%), and superoxide anion radicals (26.79%) was found at 250 µg/mL (p < 0.05 against ascorbic acid). The extract's antioxidant capacity was supported by HPTLC fingerprinting, which verified the presence of phenols, flavonoids, saponins, and terpenoids ([Thanuja et al., 2026](#)).

In addition, this plant is also capable of repairing mutations caused by insecticides such as transfluthrin (Prabhu et al., 2021). *Indigofera tinctoria* possesses anthelmintic properties resulting from the synergistic action of several compounds. Tannins and flavonoids are examples of phenolic compounds linked to pharmacological effects, such as anthelmintic activity. Furthermore, tannins have been shown to have a strong toxic effect on helminth parasites, making them a viable alternative to pharmaceutical drugs ([Rakwa et al., 2022](#)).

Indigofera tinctoria leaf extract has strong antibacterial activity against pathogens, including *E.Coli* and *S. aureus*, as well as controllable mutagenesis effects based on extract concentration. Furthermore, *Indigofera tinctoria's* high antioxidant properties demonstrate its promise in the medical area. This plant extract can considerably reduce free radicals, according to a phytochemical and HPTLC study ([Jumrah, 2025](#)).

The adaptability of *Indigofera tinctoria* is being highlighted more and more by developments in biotechnology and nanotechnology. Limited genetic variety, the effects of climate change, and competition from synthetic colors are major obstacles. However, it may be made more viable with creative agronomic techniques, residu management, and market tactics. *Indigofera tinctoria* has enormous promise as a sustainable crop that addresses both economic and environmental issues by fusing traditional wisdom with cutting edge innovation ([Rao et al., 2025](#)).

Based on these explanations, this study was conducted to determine the secondary metabolite compounds contained in the methanol extract of *Indigofera tinctoria* and its antibacterial activit.

B. Method

The study is an exploratory study that includes sample preparation, extraction, phytochemical analysis, and antibacterial activity testing of extracts from the *Indigofera tinctoria* plant.

1. Sample Preparation

Leaves from the *Indigofera tinctoria* plant were first washed under running water to remove any dirt, then cut into small pieces. Afterward, the samples were dried to reduce their moisture content and then ground into a powder using a blender.

2. Extraction (Ultrasound assisted Extraction)

Extraction is the process of separating plant bioactive fractions using extraction techniques and a system of selective solvents. Plant products are essentially liquid, semisolid, or dry powder combinations of metabolites. The specific nature of the targeted bioactive chemicals largely dictates the choice of solvent system. The solvents employed for extraction penetrate solid plant matter and solubilize molecules of comparable polarity throughout the extraction process ([Khade et al., 2023](#)).

The fundamental principle of ultrasonic extraction technology is enhancing extraction efficiency by applying ultrasound. This method utilizes the cavitation, mechanical, and thermal effects of ultrasound to accelerate the release, diffusion, and dissolution of target compounds within the cell. Cavitation occurs when high energy ultrasonic waves disrupt the medium, forming numerous microscopic cavities that collapse rapidly. The rapid collapse of these cavities generates instantaneous pressures reaching up to millions of atmospheres. This immense pressure significantly reduces the time required for cell fragmentation. The bursting of cavitation bubbles ruptures plant cell walls, facilitating rapid extraction of intracellular contents. Simultaneously, ultrasonic vibrations further enhance the release, diffusion, and dissolution of intracellular substances. These combined effects substantially improve overall extraction efficiency ([Aung & Das, 2025](#)).

In this study, ultrasonic extraction was performed by adding methanol to 100 g of dry leaves, and the mixture was extracted for 45 minutes at 50°C ([Yasser et al., 2020](#)). The resulting solution is then filtered and concentrated at 40°C using a rotary vacuum evaporator. The condensed extract is stored for further analysis.

3. Phytochemical Test

Phytochemical tests were conducted on the methanol concentrate extract. Various reagents were used, including iron (III) chloride (FeCl₃). FeCl₃ produces a green color if the sample tests positive for flavonoids. which produces a green color if the sample is positive for flavonoids. With Wagner's reagent, the formation of a brown precipitate indicates the presence of alkaloids;

Dragendorff's reagent produces an orange precipitate if alkaloids are presents. Liebermann-Burchard's reagent shows a green color for steroid compounds and red-purple color for terpenoids.

1) Iron (III) chloride (FeCl_3)

Reagent principal Iron (III) chloride in the screening of flavonoid compounds is iron ion (Fe^{3+}) and phenolic hydroxyl groups combine to form a colorless complex. Depending on the particular phenol present in the sample, the phenolic hydroxyl group interacts with ferric chloride to produce a complex that exhibits a characteristic color change. The formation will result in the presence of green flavonoid compounds ([Maheshwaran et al., 2024](#)).

2) Wagner's Test

Wagner's reagent forms a covalent bond with the alkaloid nitrogen to produce a complex precipitate of a potassium salt, which is how it screens alkaloid compounds. If alkaloid chemicals are present, the results will display a reddish-brown precipitate (Maheshwaran et al., 2024).

3) Dragendorff's Test

To screen alkaloid compounds, the Dragendorff reagent uses hydrolysis processes to control the amount of Bi^{3+} ions in solution. Acidic substances must be added to the solution to do this. Furthermore, the Bismuth (III) nitrate ion can combine with potassium iodide to form a dark brown precipitate of bismuth (III) iodide and excess potassium iodide dissolves to form potassium tetraiodobismutate. The K^+ ion and nitrogen form a covalent coordination bond. The formation of an orange, brownish orange, or light brown to yellow precipitate indicates the presence of alkaloid compounds ([Maheshwaran et al., 2024](#)).

4) Liebermann-Burchard's Test

The interaction between sterols and acetic anhydride in the presence of sulphuric acid, which results in a succession of colour changes, provides insight into the Lieberman-Burchard reagent's mode of action. The sterols undergo sulfonation, leading to the formation of conjugated dienes and trienes and the distinctive discolouration. The final shade of dark green indicates the presence of terpenes or sterols. The presence of sterols is indicated by a sequence of colour changes that often begin with pink and progress to blue, green, and finally dark green ([Maheshwaran et al., 2024](#)).

4. Antibacterial Activity Test

An antibacterial assay was performed using the agar diffusion method with a paper disc. The bacterial suspension was inoculated into the nutrient agar medium and homogenized. The mixture of media and bacteria was poured into a petri dish. A paper disc soaked with extract samples is left for 15 minutes, then placed on nutrient agar media inoculated with bacteria. The diameter of the clear zone around the disc paper was measured using a caliper after incubation for 24 hours at 37°C .

C. Results and Discussion

Indigofera tinctoria, also known as tarum or Nila, is a plant species used as a source of natural indigo dye. In addition to its use as a natural dye, this plant contains secondary metabolites that offer numerous benefits. The *Indigofera tinctoria* plant samples used in this study were first ground into a powder to increase their surface area and facilitate extraction.

1. Phytochemical Testing

Phytochemical screening identifies active compounds in plants and indicates their pharmacological potential. It is widely used on various parts of medicinal plants, including leaves, flowers, seeds, fruits, and stems. The tests were performed using several reagents, including FeCl_3 to

test for the presences of flavonoids, Dragendorff's reagent to test for the presence alkaloids, Wagner's reagent to test for the presence of alkaloids, and Lieberman Burchard reagent to identify the presence of terpenoids and steroids. The test results are shown in Table 1.

TABLE 1. Phytochemical Analysis of Methanol Extracts from *Indigofera tinctoria* Plant Leaves

No.	Reagents	Description
1	Iron (III) chloride (FeCl_3)	(+) Flavonoids
2	Wagner's reagent	(+) Alkaloids
3	Dragendorff's reagent	(+) Alkaloids
4	Liebermann-Burchard's reagent	(+) Steroids

Based on the results of the phytochemical screening presented in Table 1, the methanol extract of *Indigofera tinctoria* leaves revealed the presence of a variety of secondary metabolites, including flavonoids, alkaloids, and steroids. This is in line with research conducted by Ariyatun et al in 2022, with the results obtained, that the *Indigofera tinctoria* plant contains secondary metabolite compounds of flavonoids, steroids, and alkaloids ([Ariyatun et al., 2022](#)).

1) Flavonoids

Flavonoids are a class of polyphenolic secondary metabolites that mostly consist of flavones, flavonols, and anthocyanins. With uses in industries including food, medicine, and industry, they are essential to both human health maintenance and plant life activities. Flavonoids derived from plants can efficiently scavenge reactive oxygen species and serve as essential building blocks for the development of natural anticancer medications. The food industry frequently uses flavonoids as functional additions to enhance nutritional profiles ([Chu et al., 2026](#)).

In this study, phytochemical tests were conducted to identify secondary metabolites, particularly flavonoids, using iron (III) chloride as a reagent. The results showed that *Indigofera tinctoria* plants tested positive for flavonoids, as indicated by the formation of green color. The colour change occurs due to the formation of complexes between Fe^{3+} ions and the phenolic hydroxyl groups of flavonoids, producing coloured coordination complexes.

2) Alkaloids

Alkaloids are a type of naturally occurring compounds that often have physiological effects and include carbon, hydrogen, and nitrogen. They are often identified in blooming plants and are generally intricate structures with several types of rings. Their moniker comes from the fact that they are chemically necessary. Their role in the natural world is unclear ([Franjić, 2024](#)).

In this study, positive results were obtained in tests using Wagner's and Dragendorff's reagents, confirming the presence of alkaloids in the methanol extract of *Indigofera tinctoria*. An orange precipitate is formed by Dragendorff's reagent through the formation of a complex at the nitrogen atom of the alkaloid.

3) Steroids

Steroids have cardiogenic, insecticidal, antibacterial, and antiparasitic qualities. Additionally, steroids are a major component of herbal remedies, dietary supplements, and cosmetics. Four fused carbon rings make up the basis of steroids, which are lipid terpenoids. The rings' structure is changed when oxidized chemical groups are added to them. The oxidation of the

carbon rings itself is what causes this effect. Many steroids feature one or more hydroxyl groups, and others have one, two, or three double bonds. The presence of green or blue color indicates the presence of steroids (Luciana et al., 2024).

Testing for steroids using the Liebermann-Burchard reagent yields a positive result, indicated by the formation of a bluish green color. This reaction involves the steroid backbone by concentrated sulfuric acid under anhydrous conditions (glacial acetic acid), which is one of the main components of the Liebermann-Burchard reagent.

2. Antibacterial Activity Testing

The antibacterial activity of the methanolic leaves extract of *Indigofera tinctoria* studied through two gram-positive and two gram-negative bacteria., *E. coli* illustrated in Figure 3 and *S. typhi* illustrated in Figure 4 and the value can be seen in Table 2.

TABLE 2. The inhibition zone of *S. aureus*, *Bacillus subtilis*, *E. coli* and *S. typhi*

No.	Bacteria	Inhibition zone (mm)
1	<i>Staphylococcus aureus</i>	17.30
2	<i>Bacillus subtilis</i>	19.45
3	<i>Escherichia coli</i>	15.55
4	<i>Salmonella typhi</i>	8.80

The test results showed that the methanol extract of *Indigofera tinctoria* leaves exhibited antibacterial activity against all test bacterial isolates, including both gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative (*Escherichia coli* and *Salmonella typhi*) strains. Overall, this extract is far more effective at inhibiting the growth of gram-positive bacteria than gram-negative bacteria.

The antibacterial activity against *Staphylococcus aureus* demonstrated significant efficacy, with an inhibition zone of 17.30 mm. The presence of a large clear zone around the growth center indicates the susceptibility of this gram-positive bacterium. The inhibition zone of *S. aureus* illustrated in Figure 1.



Figure. 1. The inhibition zone of *Staphylococcus aureus*

The largest inhibition zone among all test samples, based on the data in Table 2, was 19.45 mm. The diameter of the clear zone on the *Bacillus subtilis* medium indicates that this bacterium is the most susceptible of the isolates. The inhibition zone of *Bacillus subtilis* illustrated in Figure 2.



Figure. 2. The inhibition zone of *Bacillus subtilis*

An inhibition zone of 15.55 mm indicates inhibitory activity against the gram-negative bacterium *Escherichia coli*. Although there is clear growth inhibition, its effectiveness is still lower than that against gram positive bacteria. The inhibition zone of *Escherichia coli* illustrated in Figure 3.



Figure. 3. The inhibition zone of *Escherichie coli*

The inhibitory activity against the gram-negative bacterium *Salmonella typhi* showed the lowest inhibition response compared to the other three bacteria, at only about 8.80 mm. this was

evident from the narrowest clear zone diameter around the test spot. The inhibition zone of *Salmonella typhi* illustrated in Figure 4.

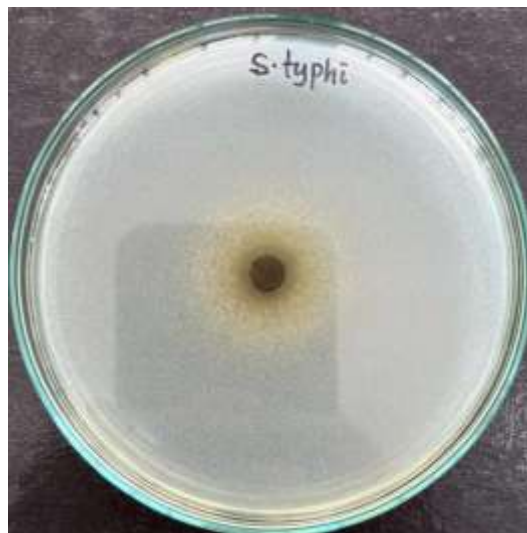


Figure. 4. The inhibition zone of *Salmonella typhi*

Differences in barrier effectiveness between gram-positive and gram-negative bacteria can be explained through structural differences in the cell walls of the respective isoates. In general, gram-positive bacteria have a simpler cell wall structure but a very thick peptidoglycan layer. However, gram-positive bacteria lack an outer membrane, so antibacterial agents can diffuse very easily through the highly permeable and porous peptidoglycan layer ([Breijyeh et al., 2020](#)). In contrast, gram-negative bacteria such as *Escherichia coli* and *Salmonella typhi* have a more complex defense system, namely in the form of an outer membrane rich in lipopolysaccharides. The outer membrane contains lipopolysaccharides and porin proteins that act as selective permeability barriers, reducing the diffusion of many antibacterial compounds into bacterial cells. This is because it is not only hydrophobicity that plays a role, but also the porin system and the selectivity of the membrane. This explains why the inhibition zone for *Salmonella typhi* is the smallest, at only 8.80 mm.

This significant antibacterial activity is closely related to the presence of secondary metabolites detected in phytochemical assays. Flavonoids may exert antibacterial activity through multiple mechanisms, including disruption of membrane permeability, inhibition of nucleic acid synthesis, and interaction with membrane proteins via hydrogen bonding. The phenolic groups (-OH) of flavonoids can form hydrogen bonds with membrane proteins. This can disrupt enzyme function and membrane integrity. Then alkaloid compounds that have nitrogen components that can interfere with the constituent components of peptidoglycan in the bacterial cell wall, which causes the cell wall layer to be formed in an incomplete manner and trigger cell death (lysis), and steroid compounds that can interact with cell membrane phospholipids that are permeable to lipophilic compounds, causing leakage in the integrity of bacterial cells.

Compared with the findings of [Hosain et al. \(2024\)](#), which showed that all bacteria strains tested exhibited inhibition zones of varying sizes. The largest clear zones were observed for *Staphylococcus aureus* (10 mm), *E. coli* (5-7 mm) and *Salmonella typhi* (2-3 mm) ([Hosain et al., 2024](#)). The inhibition zones observed in the present study were larger, suggesting that differences in extraction methods, solvent polarity, phytochemical composition, or extract concentration may influence antibacterial potency.

D. Conclusion

Indigofera tinctoria plant contains several groups of secondary metabolite compounds, namely flavonoids, alkaloids, and steroids. In addition, the plant extract proved effective in inhibiting the growth of gram-positive and gram-negative bacteria but was particularly effective against gram-positive bacteria. Based on the diameter of the clear zone, the highest effectiveness was observed with *Bacillus subtilis* (19.45 mm), followed by *Staphylococcus aureus* (17.30 mm), *Escherichia coli* (15.55), and the lowest with *Salmonella typhi* (8.80). This antibacterial activity is related to secondary metabolites contained in it, namely flavonoids that denature cell proteins, alkaloids that interfere with peptidoglycan synthesis, and steroids that damage the integrity of bacterial cell membranes. In addition, further research into the therapeutic properties of *Indigofera tinctoria* is highly recommended.

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