STUDIES ON ANTIMICROBIAL PROPERTIES OF *WITHANIA COAGULANS* AND *WITHANIA SOMNIFERA*

Dissertation submitted in partial fulfillment of the requirement for the degree of

Master of Science

In

Microbiology

By

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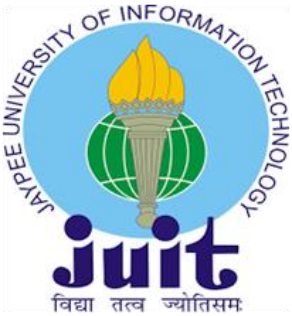
Under the supervision

of

Dr. Rahul Shrivastava

to



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DECLARATION

I hereby declare that the work presented in this report entitled "**Studies on Antimicrobial Properties of *Withania coagulans* and *Withania somnifera***" in partial fulfilment of the requirements for the award of the degree of **Master of Science in Microbiology** submitted in the **Department of Biotechnology & Bioinformatics**, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from July 2024 to May 2025 under the supervision of **Prof. (Dr.) Rahul Shrivastava,** Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

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CERTIFICATE

This is to certify that the work reported in the M.Sc. Microbiology thesis entitled **"Studies on Antimicrobial properties of *Withania coagulans* and *Withania somnifera*"** which is being submitted by **Ms Sakshi sharma (235112001) in** fulfilment for the award of Masters of Science in Microbiology by the Jaypee University of Information Technology, is the record of candidate's own work carried out by her under my supervision. This work is original and has not been submitted partially or fully anywhere else for any other degree or diploma.

Dr. Rahul Shrivastava
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Abstract

Plant alternatives to synthetic antibiotics are being investigated as a result of the emergence of antimicrobial resistance. The present study investigates the antimicrobial activity of fruit extracts of *Withania coagulans* and *Withania somnifera* against *Escherichia coli* and *Bacillus subtilis*. Four solvents were used to extract fruits: methanol, ethanol, acetone, and distilled water. The presence of significant secondary metabolites, including alkaloids, flavonoids, saponins, tannins, and glycosides, was verified by phytochemical screening. The extract's antibacterial efficacy was evaluated using the disc diffusion method. the methanolic and ethanolic extracts of the tested Solvents demonstrated comparatively higher activity, particularly against *B. subtilis.* The findings demonstrated that the presence of phytochemicals with potential for therapeutic use in *Withania* fruits contributes to its significant antibacterial activity. The results of the current study recommend further research to isolate and characterize the active compound responsible for antimicrobial activity and support the use of these plants in traditional medicine to treat infections.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

For hundreds of years, ancient medicines have used therapeutic plants as the basis of their treatments. These plants contain a huge number of bioactive chemicals that are very important for healing. *W.coagulans* (cheese maker) and *W. somnifera* (Ashwagandha) are two of them that are well known for their ability to kill bacteria. [1].

Withanolides, alkaloids, and phenolic compounds are thought to be responsible for their pharmacological effects. The antimicrobial activity of *W. coagulans* was due its ability to kill both gram-positive and gram-negative bacteria, as well as fungi by breaking down microbial membranes and stopping important enzyme activity.

W. somnifera exhibits broad-spectrum antimicrobial activity, which is usually related to its withanolide content, thus a potential against antibiotic-resistant microorganisms. Withanolides and Alkaloids are the bioactive phytochemicals which interfere, disrupt microbial cell membranes, causing to leakage of cellular contents. Tannins and flavonoids inhibit microbial enzymes and also inhibit crucial metabolic. Ashwagandha contains withanolides, saponins, phenolic acids, and alkaloids, which are responsible for its therapeutic activity. Withanolides inhibit bacterial cell walls and block biofilm formation. Saponins and phenolics are the inhibitory enzymes that blocks microbial growth and replication [2].

CHAPTER 2

LITERATURE REVIEW

2.Literature review

The plant family Solanaceae, consisting of approximately 20 species of small trees and shrubs and *Withania* genus is included to it [1]. Even though these plant types originally belong to the Indian subcontinent, they have also migrated to the regions of Africa and the Middle East through the centuries. Two of the most widely used species among the other species are *Withania somnifera* (Ashwagandha) and *Withania coagulans* ("Indian cheese maker", Indian rennet, Panir ke phool, or Panner Doda) because they find wide applications Ayurveda and traditional medicine [3].

2.1 Withania somnifera (Ashwagandha)Historical Background:

Withania somnifera has a vast historic use in Ayurvedic medicine, where it is often referred to as "Ashwagandha" (which translates to "smell of the horse" in Sanskrit). This name is said to derive from the plant's ability to impart strength, much like that of a horse. The root of the plant is primarily used in traditional remedies, although its berries and leaves have also been used in some cultures. Its use in traditional medicine can be traced back over 3,000 years, where it was considered one of the most important herbs in Ayurvedic healing practices [3]. In Sanskrit texts, "Ramayana' is a group of herbs called *Withania somnifera* that are thought to help people live longer and feel better in general in the past, people treated a wide range of problems, such as stress, insomnia, joint pain , anxiety, inflammation. Using it also improved memory and cognitive function, as well as strength and stamina. Cultural and traditional use: ayurvedic doctor gave Ashwagandha to people to help them balance their three doshas: vata pitta, and kapha. People thought it calmed, nourished and refreshed the nervous system and they use it to clear their minds and fight the effects of stress. In Unani medicine, Ashwagandha was known for its healing power. [4].

Modern-Day Relevance:

In the modern era, *Withania somnifera* is extremely popular today and used everywhere across the globe, ranging from tablets and capsules to powders and teas in most supplements. It has been extremely emphasized adaptogenic properties, particularly for its ability to help the body

deal with stress, anxiety, and fatigue. A lot of the use of *Withania somnifera* has also been confirmed by modern science. Studies have proven that the herb to be able to reduce cortisol levels, improve the quality of sleep, and improve global cognitive and immune function. It has also been researched for neuroprotection, anti-inflammatory, and anticancer activity [5].

2.2 Withania coagulans (Indian Cheese Maker)

Withania coagulans is not as well known as *Withania somnifera*, but it is still an important part of traditional medicine and food in India and nearby areas. People called it 'Indian cheesemaker' because it was used to make cheese. People have used this plant to make cheese for hundreds of years. People have used this coagulating effect for hundreds of years to make dairy products by coagulating milk. The plant itself found in rural farming communities where dairy production is common. *W. coagulans* has round, green fruit. It is thought to be a useful plant for both cooking and medicinal purposes. It has been used in ayurvedic medicines to help to deal with the ailments like digestion and respiratory problems. [6].

Cultural and Traditional Use:

Ayurvedic and folk medicine, *Withania coagulans* was used to treat digestive problems like constipation. The herbal mixtures helped with digestion and gut health. People used the fruit of *Withania coagulans* to curdle the milk when making cheese in the past. This was especially important in places where refrigeration and advanced dairy technologies were not available. [7].

Relevance Today:

Although *W.coagulans* is not as well studied as *Withania somnifera*, It is becoming more well known for its therapeutic benefits due to recent studies on its anti-inflammatory, antioxidant, and antibacterial qualities. Additionally, its use as a natural coagulant in the dairy industry has raised interest in environmentally friendly, chemical free substitutes for the synthetic coagulants frequently used in the making of cheese [7].

Both *Withania somnifera* and *Withania coagulans* are the important herbs of ancient times have rich histories and important roles in traditional healing systems, particularly in Ayurveda. *Withania somnifera*, has some adaptogenic, supporting mental clarity, reducing stress, and

improving physical health. It is a key herb in the Ayurvedic pharmacopeia and has become widely used in the modern wellness industry [8].

Conversely, *Withania coagulans* is slightly more specific but no less essential, and is used primarily for gastrointestinal health, and coagulating milk in cheese-production. Although it has not been as widely recognized internationally as Ashwagandha, its characteristics are under investigation for more general health use, as a possible antibacterial, anti-inflammatory, and digestive-supportive activities [9].

Table 2.1. Comparative Analysis of Withania *coagulans* and *Withania somnifera*

Property	***Withania coagulans***	***Withania somnifera***
Primary Compounds	Withanolides, alkaloids, tannins	Withanolides, saponins, phenolic acids
Spectrum of Activity	Broad-spectrum (bacteria and fungi)	Broad-spectrum with higher antifungal focus
Mechanism	Disrupts membranes, inhibits enzymes	Disrupts walls, biofilm prevention
Application Areas	Digestive and respiratory infections	Skin and systemic infections

2.3 Phyto-chemicals

There are researchers who have thoroughly studied *Withania* species, which have resulted in the identification, characterization, and isolation of bioactive compounds in various plant components [10]. It contains alkaloids, flavonoids, tannins, and a number of steroidal lactones. In the chemical and spectral data, ten new phytoconstituents were isolated from methanol-extracted, air-dried *W. coagulans* fruit. *W. coagulans* constituents of varied nature were investigated in three extracts: methanolic, hydroalcoholic, and chloroform [11]. According to reports, the methanolic extract had higher levels of total phenolic content (55.9 mg/g), total tannins (76.6 mg/g), total flavonoids (0.88 mg/g), and total flavanol (0.25 mg/g) than the hydroalcoholic and chloroform extracts. Some bioactive compounds are as follows:

2.3.1 Withanolides

Withanolides are a group of naturally occurring C-28 steroidal lactones predominantly found in the *Withania* genus of the Solanaceae family, particularly in *Withania somnifera* (commonly known as Ashwagandha) and *Withania coagulan* (commonly known as Indian cheese maker or Paneer Dodi) [12]. These bioactive compounds are structurally similar to ergostane-type steroids and are responsible for a wide range of therapeutic activities. Over 40 withanolides have been isolated from *W. somnifera*, including withaferin A, withanolide A, withanone, and withanoside IV and V, many of which have demonstrated broad-spectrum antimicrobial, anti-inflammatory, antioxidant, and anticancer properties [13].

The antimicrobial potential of withanolides is well documented. For example, withaferin A has been shown to destabilize bacterial cell membranes and to suppress biofilm formation in pathogens like *Staphylococcus aureus* and *Escherichia coli*. Likewise, extracts with withanolides in *W. coagulans* demonstrated inhibitory activity against fungal strains such as *Candida albicans* and *Aspergillus niger*. These compounds are believed to be responsible for their ability to interact with microbial enzymes, membrane integrity, and quorum sensing pathways. Additionally, withanolids could synergize with the standard antibiotics, and hence lower resistance development and improvement of efficacy [14].

Because of their steroid-like structure, withanolides also control human immune functions presumably aiding the host in resisting infections. Their ability to penetrate cross cell membrane and target intracellular sites gives them a selective benefit in antimicrobial therapy. Moreover, some studies have shown that the withanolide-enriched extracts of *Withania somnifera* and *Withania coagulans* have very low cytotoxicity against human cells and are thus good candidates to become natural antimicrobial agents [15].

2.3.2 Alkaloids

Alkaloids constitute a vast and diverse group of naturally occurring organic compounds that possess contain one or additional nitrogen atoms, usually derived from amino acids. They are also renowned for their extremely potent biological and pharmacological characteristics, including antimicrobial, antifungal, antiviral, and anticancer activities. In the case of the *Withania* species, both *Withania somnifera* and *Withania coagulans* is a rich source of alkaloids like anaferine, tropine, cuscohygrine. Alkaloids are mostly responsible for the medicinal uses traditionally ascribed for these species[15].

The ability of the alkaloids to disrupt microbial DNA replication, protein synthesis, and cell membrane stabilization accounts for a significant portion of their antimicrobial activity. Aneurine, the main alkaloid of *W. somnifera,* for example has been shown to have antibacterial properties through the disruption of cell walls and the inhibition of nucleic acid synthesis. Furthermore, studies have shown that alkaloid-rich extract of *W. coagulans* can stop the growth of common bacterial pathogens like *Pseudomonas aeruginosa, Staphylococcus aureus,* and *E.coli* as well as fungal species like *Candida albicans* [16].

Besides, the synergistic effect of the alkaloids with other phytochemicals such as withanolids and flavonoids is the cause of increased antimicrobial activities of the plant extract for their low cytotoxicity toward human cells and owning to their high selectivity towards microbial cells[17]. They are potential lead compounds for the development of natural antimicrobial medicines. With increasing resistance towards conventional antibiotics, research on alkaloids of *Withania* species offers a prolific platform for the identification of new therapeutic agents that can overcome multi drug resistance and serve as alternatives to synthetic antibiotics [18].

2.3.3 Saponins

Saponins are a group of naturally occurring glycosides in most medicinal plants, example *Withania somnifera* and *Withania coagulans*. They are structurally, a sapogenin attached to one or more sugar chains[19]. They are very renowned for their surface like activities, giving them the ability to produce soap like bubbles when in water. Apart from that saponins have a myriad biological process, including antimicrobial, antifungal, anti-inflammatory, and immunomodulatory activities[20].

The antimicrobial activity of saponins is largely due to their capacity to interact with and alter microbial cell membranes. The membrane-permeabilizing action results in enhanced cell leakage of cellular contents and eventual cell lysis. Experiments have indicated that saponin-rich extracts of *W. somnifera* and *W. Coagulans* are active against both Gram-positive and Gram-negative bacteria, as well as fungal pathogens. For instance, saponins in *W. somnifera* have been found to suppress the growth of *S. aureus* and *C. albicans,* indicating a general antimicrobial activity [21].

In addition, saponins could also increase the efficacy of other bioactive compounds like alkaloids and withanolides present in these plants through synergistic effects, thus adding to the overall antimicrobial activity of the extracts. Their low toxicity to human cells and plant origin makes them attractive for the development of plant-based antimicrobial products. The

fact that *W. somnifera* contain saponins validates their use in treating infections based on traditional practices and strengthens their promises as alternative antimicrobial agents to fight antimicrobial resistance [22].

2.3.4 Flavonoids

A broad class of phenolic chemical found in the kingdom of plants are known as flavonoid and are renowned for their significant pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, and anticancer properties. Both *W. somnifera* together with *W. coagulans* were found to have several flavonoids, including kaempferol, quercetin, and isorhamnetin, which are responsible for their pharmacological and folk medicinal applications. These compounds are crucial elements of the plant defence mechanism and also in the instance of most of the health-supportive effects as reported in humans [23].

The antimicrobial activity of the flavonoids is primarily rooted in their capacity to interfere with microbial enzymes, destabilize cellular membranes, inhibit nucleic acid synthesis, and inhibit energy metabolism. Flavonoids can also chelate metal ions essential for microbial cells, eventually resulting in death [23]. It has been found through experiments that flavonoid-rich extracts from *W. somnifera* and *W. coagulans* exhibit bactericidal and fungicidal activities against a broad range of disease-causing organisms such as *E. coli*, *P. aeruginosa, S. aureus,* etc[24].

Furthermore, flavonoids also can synergistically interact with other phytoconstituents such as alkaloids, withanolides, and saponins, enhancing the overall antimicrobial activity of the plant extracts. Their natural origin, low toxicity, natural origin, and multifunctional bioactivity make them promising candidates for the creation of new plant-based antimicrobial medicines. The existence of flavonoids in *Withania* species not only validates their traditional medicinal use but also emphasizes their promise in solving the newly emerging problem of antimicrobial resistance[25].

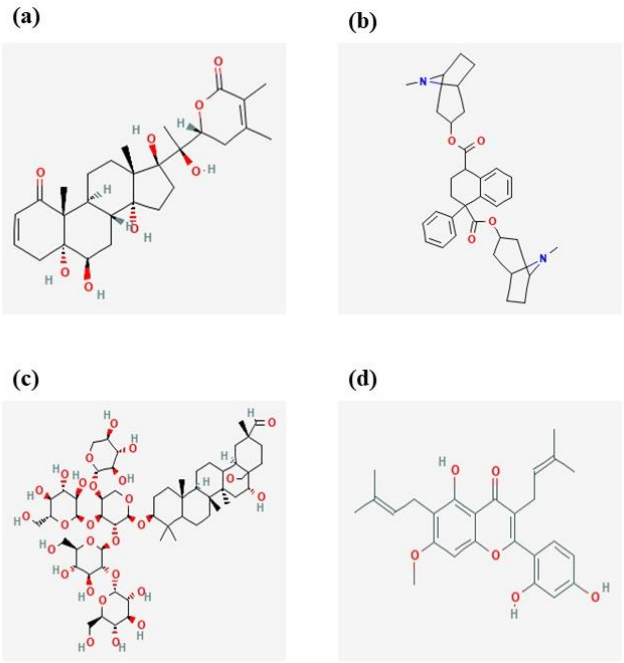


Fig 2.1: Chemical structures of (a) Withanolides (b) Alkaloids (c) Saponin (d) Flavonoid

2.4 Different anti-microbial effects

Withania somnifera is found to possess antimicrobial properties against several bacterial species such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*, as well as against some fungi and viruses. The antimicrobial properties of *Withania somnifera* are commonly explained on the basis of the combined effects of its withanolides, alkaloids, and saponins [25].

It is especially active against Gram-positive bacteria relied on its capacity to interfere with the bacterial cell membrane integrity and stop cell wall synthesis. Extracts of *Withania somnifera* have also been found to possess notable antifungal activity, especially towards organisms such as *Candida albicans* [26].

Mechanisms of Antimicrobial Action

The antimicrobial activities of both *Withania coagulans* and *Withania somnifera* are due to some important mechanisms of action:

Membrane Disruption

Withaferin A, one of the major bioactive compounds in *W. somnifera* (Ashwagandha), showed extensive antibacterial activity, especially against *P. aeruginosa,* a drug-resistant pathogen. In a study, withaferin A exhibited strong antibacterial activity against *P. aeruginosa.* The researchers used membrane stabilization assays and electron microscopy to study the mechanism of action. Their research showed that withaferin A degrades the integrity of the bacterial cell membrane, creating enhanced permeability and ultimately cell lysis. This is the major aspect of its bacterial action. There was also a noted enhancement in antioxidant enzymes like superoxide dismutase and catalase, along with diminished macrophage localization, reflecting a general decrease in the inflammatory response[27]

Enzyme Inhibition

Quercetin and Kaempferol are flavonoids whose antimicrobial activities can be partly explained by their capacity to disrupt microbial enzymatic processes crucial for survival and growth. Both of them have been demonstrated to suppress enzymes essential for fatty acid biosynthesis in bacteria, including 3-oxoacyl-[acyl carrier protein] reductase (FabG) and enoyl-acyl carrier protein reductase. These are essential enzymes required for the construction of are vital for the formation of the bacterial cell envelope. Inhibition of these enzymes interferes with the synthesis of fatty acids, resulting in impaired, compromised cell membrane integrity and functionality in pathogens. Quercetin has been reported to inhibit bacterial DNA gyrase and topoisomerase IV, DNA replication and transcription enzymes that are critical for bacteria. Quercetin inhibits the binding of these enzymes, thereby interfering with the supercoiling and relaxation of DNA, and ultimately the bacterial cell division and growth [28]

Inhibition of Biofilm formation

Kaempferol has been found to inhibit *S. aureus* biofilm development by suppressing the genes involved in adhesion and biofilm formation, including clfA, clfB, fnbA, fnbB, sarA. The suppression prevents the formation of chronic infections that are biofilm related [29].

Synergistic effects of Phytochemicals

The synergy of numerous bioactive compounds present in *Withania* species may cause synergistical antimicrobial activities. For instance, the presence of flavonoids may increase the

microbial cell membrane permeability, enabling other compounds such as withanolids and alkaloids to penetrate and exert their antimicrobial activities more efficiently[30].

The antimicrobial properties of *Withania coagulans* and *W. somnifera* are attributed to their bioactive constituents, which include flavonoids, alkaloids, saponins, withanolides. They are highly qualified for further research as natural antimicrobial agent due to their ability to disrupt microbial cell memberanes. Obstruct metabolic processes, and reverse oxidative stress. Both species are good substituent for artificial antibiotics. Especially in light of the escalating issues with antibiotic resistance. To completely comprehend the precise mechanism of action determine their therapeutic potential in clinical use, more research is required. [29].

Both *W. coagulans* and *W. somnifera* both have a wealth of bioactive compounds that are accountable for their antimicrobial actions. These includes, alkaloids, saponins, and flavonoids, which are all accountable for the capacity of plant to fight a range of bacterial, fungal, and viral infections. In recent years, the antimicrobial effects of these compounds have been, in the recent years, which has shown there potential to be used as natural alternatives to synthetic antibiotics given that resistance to traditional medicines is on the rise [30].

2.5 Antibacterial Activity

Extracts of *W. somnifera* have been reported to be active against a number of bacterial pathogens, such as Gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis* and Gram-negative bacteria*: Escherichia coli, Pseudomonas aeruginosa.* Antibacterial activity is the capacity of an agent to inhibit the growth of or kill bacterial pathogens. *W. coagulans* also demonstrates promising antibacterial potential, although less investigated than *W. somnifera* [31].

2.6 Antiviral Potential

Medicinal plants like *W. somnifera and W. coagulans* are rich in phytochemicals that possesses biological actions such as antiviral activities.

W. somnifera has been extensively studied for its broad-spectrum antiviral potential. Withaferin A has shown inhibitory activity against viral replication by targeting key viral enzymes (e.g. viral proteases and polymerases). *W. somnifera* enhances host immunity, particularly through activation of natural killer cells and macrophages. This indirect effect helps the body better defend against viral infections, reducing viral load and disease progression. Some flavonoids

in *W. somnifera* may block viral attachment to host cells by binding to surface glycoproteins or host receptors [31].

2.7 Applications of *Withania coagulans* and *Withania somnifera*

Both *Withania coagulans* and *Withania somnifera* are celebrated for its extensive medicinal applications:

Antimicrobial Agents: They exhibit potent antibacterial, antifungal, and antiviral activities, making them useful in treating infections, particularly in drug-resistant pathogens.
Anti-inflammatory Properties: *W. somnifera* is utilized to treat arthritis and other inflammation-related diseases. *W. coagulans* also helps alleviate inflammation in chronic conditions such as diabetes and cardiovascular disorders[32].
Stress and Cognitive Health: *W. somnifera* is renowned for its adaptogenic properties, reducing stress, enhancing memory, and potentially preventing neurodegenerative diseases like Alzheimer's and Parkinson's [33].

2.7.1 Management of Metabolic and Endocrine Disorders

Diabetes Treatment: *W. coagulans* possesses hypoglycemic activity, decreasing blood glucose and insulin sensitivity.
Hormonal Balance: *W. somnifera* controls the endocrine system, treating thyroid imbalances, adrenal exhaustion, and reproductive hormone imbalances.
Weight Management: with improved metabolism and reduced stress-related overeating, *W. somnifera* provides healthy weight maintenance [34].

2.7.2 Cancer and Immune Support

Antitumor Activity: Both plants have withanolides that trigger apoptosis in cancer cells but are not active against normal cells. They are there a potential drug in adjunct cancer therapy while sparing healthy cells. This makes them promising candidates in complementary cancer therapy.
Immunomodulatory Effects: These species strengthen immune responses, which is beneficial in combating infections and autoimmune diseases [35].

2.7.3 Cardiovascular Benefits

Cholesterol and Lipid control: *W. coagulans* reduces LDL cholesterol and triglycerides, Promoting cardiovascular well-being.

Antioxidant Protection: Both plants combat oxidative stress, preventing atherosclerosis and related cardiovascular diseases [36].

2.7.4 Applications in Traditional and Modern Medicine

Digestive Health: In traditional medicine, *W. coagulans* has been employed to treat liver diseases, enhance digestion, and stabilize bile secretion.
Respiratory diseases: The two plants help treat Bronchitis, asthma, and respiratory infections and also help in managing asthma, bronchitis, and respiratory infections.
Skin and Cosmetic applications: Due to their antioxidant activity, they find applications in cosmetic products, wrinkles, enhance the texture of skin, and promote wound healing.

Industrial and Future Applications

Pharmaceutical Use: both forms are employed in medications for anxiety, chronic pain and effective disease.
Agriculture: Since they are antimicrobial properties, they are studied as natural pesticides.
Nutritional Supplements: Both of them are utilized in dietary supplements for total health, the immune system, and stress.
Biotechnological Research: Scientists are researching these plants to isolate bioactive compounds in order to create synthetic analogs for the production of drugs [36].

CHAPTER 3

Materials and Methodology

3.1 Materials

3.1.1 Microbial strains

Strain	Sources
E. coli DH5α	IMTECH (Institute of Microbial Technology), Chandigarh, India
Bacillus subtilis	IMTECH (Institute of Microbial Technology), Chandigarh, India
Mycobacterium smegmatis MC^2155	Central Drug Research Institute (CDRI), Lucknow, India
Aspergillus niger	JUIT Repository

3.1.2 Media

Nutrient Agar	HIMEDIA
Nutrient Broth	HIMEDIA
Luria Broth	HIMEDIA

3.1.3 Chemicals

Crystal violet	Loba Chemie
Gram's Iodine	HIMEDIA
Safranin	HIMEDIA
Basic fuschin	HIMEDIA
Methylene Blue	Fisher Scientific
Lactophenol Cotton Blue	Loba Chemie
Glycerol	Merck
Agarose	HIMEDIA
Antibiotic Powder of Ampicillin	HIMEDIA
Sodium Chloride	Merck
SDS	BioRed
Saturated Phenol	SRL
Chloroform	SRL
Isomyl	SRL

3.1.4 Instruments

Weighing Balance	Citizon
Autoclave	REFLITECH
Incubator	Thermo Scientific
Incubator shaker	Macflow Engineering
Laminar Air Flow	Rescholar
pH meter	Eutech
Centrifuge	Eppendorf
Vortex mixer	REMI
Water Bath	NSW INDIA
Light microscope	OLYMPUS
4°C storage	Allied frost
-20°C storage	Blue Star

3.2 Methods

3.2.1 Different Microbiological Techniques

3.2.1.1 Simple streaking

1. Nutrient agar media was poured on to sterilized petri plate and labelled them properly
2. The inoculation loop was sterilized till red hot on the flame of a Bunsen burner.
3. After cooling down of loop isolated colony of bacteria was picked by loop from cultured plate.
4. The loop was dragged gently on the nutrient agar plate in a zig-zag pattern.
5. The loop was again sterilized until it became red hot.
6. At last petri plates was kept in incubator for overnight at 37°C.

3.2.1.2 Quadrant Streaking

1. Nutrient agar media was poured on to sterilized petri plate and labelled them properly
2. The inoculation loop was sterilized till red hot on the flame of a Bunsen burner.
3. After cooling down of loop isolated colony of bacteria were picked by loop from cultured plate.
4. Smear was prepared at one end of petri plate and dragged to the other end.
5. The loop was again sterilized until it became red hot.
6. The plate was rotated 90 degrees and other streaks were made from the end of the previous streak.

7. The petri plate was labelled as four different quadrant and the technique of dragging from one end to other where last streak was left.
8. Similar procedure was repeated for 3 times.
9. In last step petri plate was incubated for overnight at 37°C.

3.2.1.3 Gram staining

1. A clean glass slide was prepared.
2. One drop of autoclaved distilled was kept on the slide and a thin smear was prepared by taking culture from tube by inoculation loop.
3. Slide was kept for air drying and fixed with heat properly over Bunsen burner.
4. Crystal violet dye was poured on slide and slide was kept for about 45 seconds to 1 minute and then washed with tap water gently.
5. Gram's iodine mordant dye was poured on slide and kept for 1 minute and again rinsed with tap water.
6. Then slide was washed with decolorized that was 95% alcohol for about 35 seconds, the slide was once more rinsed with water.
7. Counterstain safranin dye was poured and kept for about 40 seconds and washed with tap water.
8. Slide was kept for air drying properly.
9. After that slide was observed under the light microscope[37]

3.2.1.4 Acid fast staining

1. A clean glass slide was prepared.
2. One drop of autoclaved distilled was kept on the slide and a thin smear was prepared by taking culture from tube by inoculation loop.
3. The slide was fixed with heat properly inside laminar air flow.
4. After the slide get dried, slide was paced on wire gauze and hot plate.
5. Flooded the smear with carbol fuchshin.
6. Slide was rinsed with running tap water gently.
7. Slide was washed with decolourizer (3% conc. HCl in 96% Ethanol) and then rinsed with water.

8. Cell was counterstained with Methylene Blue for about 2 minutes.
9. Slide was washed with tap water again.
10. The slide was kept for air drying.
11. After that slide was observed under the light microscope.

3.2.1.5 COLONY FORMING UNIT

1. Sample was taken in sterilized test tube from campus from soil.
2. In test tubes 9ml of autoclaved water was taken in each tube.
3. 1ml sample was taken from the first test tube and transferred to the second tube and then in all the tubes were serially diluted simultaneously, for example: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}
4. From each tubes including neat 200µl of sample was spread on different plates containing nutrient agar.
5. Sample was uniformly spread on the plates with the help of L shaped spreader.
6. Plates were kept in an incubator at 37℃ for 24 hours.
7. After 24 hours, colony was counted from plates and CFU was calculated.

3.2.1.6 Genomic DNA isolation from *E. coli*

1. *E. coli* sample was inoculated in Luria broth and kept for incubation for 24 hours at 37℃.
2. After 24 hours the *E. coli* culture was transferred to the 2ml Eppendorf tubes and centrifuged for five minutes at 12000 rpm at room temperature.
3. The Supernatant was discarded and the pellet was kept.
4. 500µl of extraction buffer and 50 µl was added to the pellet and mixed by vortex.
5. After mixing the tubes were incubated at 65℃ for thirty minutes in water bath.
6. Tubes were kept in water bath till the sample get cleared.
7. After incubation 550µl of Phenol and Chloroform (1:1) was added into the tubes.
8. Tubes were centrifuged at 10,000rpm, for five minutes at Room temperature. Two layers was formed.
9. The upper layer (Aqueous layer) was transferred to the fresh Eppendorf tubes.

10. Took 400 µl of sample from aqueous layer in other Eppendorf tube of 1.5ml.
11. To the tubes intended for the precipitation of DNA, double volume of cold isopropanol was added with 40 µl of 5M NaCl.
12. Tubes were kept at -20℃ for 20 hours.
13. Tubes were again centrifuged at 12000 rpm for fifteen minutes at 4℃.
14. The supernatant was discarded and the DNA pellet was rinsed with 1ml of 70% ethanol.
15. Eppendorf tubes were centrifuged again at 12000 rpm for five minutes at 4℃.
16. DNA pellets kept for air drying by inverting the tubes.
17. The pellet was dissolved in nuclease free water.
18. After those tubes were stored in 4℃ refrigerators.
19. The 0.8% Agarose was prepared for gel electrophoresis
20. 1µl Ethidium bromide was added to the agarose.
21. The gel was kept in an electrophoresis chamber and 1X TAE was added.

3.2.2 EXTRACT PREPERATION

a. To conduct phytochemical screening, collected plant leaves (*Withania somnifera)* and wet weighed the leaves and ground them in a mortar pestle.
b. For *Withania coagulans* dry weight of its fruit was measured and then crushed in the grinder.
c. In a falcon tube, 1g of powder or paste was dissolved in 10 ml of each of four distinct solvents: ethanol, water, acetone, and methanol
d. Falcon tubes were then placed in a shaking incubator at room temperature for 24 hours.
e. Filter the liquid extract through filter paper and store the residue in cold storage for future use. The extract is then concentrated using a rotary evaporator at their boiling points.
f. And reconstituted the extract in methanol, but the water extract was reconstituted by water itself. Now these can be used for further tests.[36]

3.2.3 Preliminary Qualitative Test:

(1) Ferric Chloride Test (for Tannins) Place 1milliliter of plant extract and 0.5% ferric chloride in a test tube. Tannins are identified by the appearance of a brownish-green or blue-blacktint.

(2) Salkowski test (glycosides): In a test tube, add 1ml plant extract, 2 mL concentrated sulphuric acid from the side wall, and 2ml chloroform. Glycosides are distinguished by the appearance of a reddish-brown blue.

(3) Dragendorff's test (alkaloid): 1ml of plant sample was placed in a test tube and 2-3 drops of Dragendorff reagent were added. Turbidity or precipitation indicates the presence of alkaloids.

(4) Froth test (for saponins): Shake a 2ml plant sample with distilled water in a test tube. The presence of foam implies the presence of saponins.

(5) Salkowski test (for triterpenoids): Placed one ml of plant extract in a test tube and mixed it with 2 ml of concentrated sulfuric acid (along the test tube's wall) and 2 ml of chloroform. Triterpenoids are identified by the appearance of a reddish-brown hue near the contact.

(6) Alkaline reagent test (flavonoids): Mix 2ml of plant extract with 3-4 drops of sodium hydroxide. Then a dark yellow cooler will develop. Then, droplets of diluted HCl are added. If it turns colourless, it shows the presence of flavonoids.

Then test tube was heated at 40 degrees Celsius and incubated for 5-10 minutes. The appearance of blue collar indicates the presence of proteins.

(7) Molisch test (or carbohydrates): Took I ml of plant extract and 1-2 drops of polish reagent added and then mixed with adding H2S04. Carbohydrates are indicated by the development of violet/purple/blue ring in between the layers.

(8) Ferric chloride test (for coumarins): Took 2ml of plant extract and few drops of ferric chloride solution. There must be formation of deep green colour. Then added conct. HNO3. The appearance of yellow colour indicates the presence of coumarin**.**

(9) Bontrager's test (for quinones): 2ml plant extract combined with some (concentrated) sulphuric acid (nearly the test tube's wall). Quinones are identified by the formation of a red colour.

(10) Ferric chloride test (phenols): Iml of plant extract was combined with 3-4 drops of 1% ferric chloride and 0.5ml of potassium ferrocyanide. Phenols are identified by the appearance of a greenish-blue tint.

(11) Ninhydrin test (for proteins): Mix 1ml plant sample with 0.2% ninhydrin.

(12) Molisch test (or carbohydrates): Add 1 mL of plant extract, 1-2 drops of polish reagent, and mix with H2S04. Carbohydrates are identified by the formation of a violet/purple/blue ring between the layers.

(13) Ferric chloride test (for coumarins): Used 2ml of plant extract and a few drops of ferric chloride solution. A deep green colour must be formed. Then, add the concentrated HNO3. The appearance of yellow suggests the presence of coumarin[36] [32].

3.2.4 Antimicrobial sensitivity test (AST)

The antimicrobial activity of *W. coagulans* and *W. somnifera* fruit extracts was evaluated using the Agar Well Diffusion Method against *Escherichia coli* and *Bacillus subtilis*. Four different solvents—methanol, ethanol, acetone, and distilled water—were used for extraction.

Plant fruit materials were shade-dried and grinded into fine powder. A 1:10 (w/v) ratio was used, i.e., 5 grams of plant powder was soaked in 50 mL of each solvent. The mixtures were incubated at room temperature for 48–72 hours with occasional shaking.

After extraction, the solutions were filtered using Whatman No. 1 filter paper.

The filtrates were then evaporated using a water bath at 40–50°C to obtain crude extracts, which were stored at 4°C for further use[39].

Preparation of Bacterial Cultures

Pure cultures of *E. coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive) were obtained and grown on nutrient agar plates.

A loopful of bacteria was inoculated into Luria broth, nutrient broth and incubated at 37°C for 18–24 hours. The turbidity of the bacterial suspension was adjusted to match 0.5 McFarland standard (~1.5 × 10^8 CFU/mL).

Agar Well Diffusion Method

Sterile nutrient agar plates were prepared and inoculated with the standardized bacterial suspension using a sterile cotton swab to ensure uniform lawn culture.

Wells of 20 mm diameter were punched into the agar using a sterile borer. Each well was filled with 40 µL of the respective plant extract.

Positive controls used were standard antibiotics (e.g., ampicillin or ciprofloxacin), while the respective solvents served as negative controls to rule out solvent effect [39].

CHAPTER 4

RESULT AND DISCUSSION

RESULTS

4.1 DIFFERENT MICROBIOLOGICAL TECHNIQUES

a. Simple streaking

Culture of *E. coli* is streaked on NA (nutrient agar). Representative image of culture plate [fig.4.1(a)].

b. Quadrant streaking

Quadrant streaking of *E. coli*, *B. subtilis*, and *M. smegmatis* was done to obtain isolated colonies. And these colonies were used for further experiments. Representative images of culture plate [fig.4.1, (b)].

c. Gram Staining

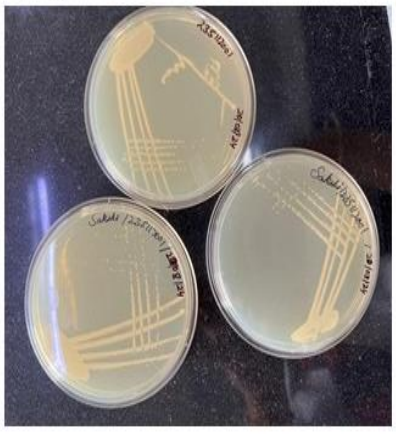
Gram staining was done to confirm that the colonies we have isolated by streaking contained pure culture of *E. coli* and *B. subtilis.* Representative image of gram staining [fig4.1(c, d)]

d. AFB Staining

AFB staining detect acid fast bacteria like *Mycobacterium tuberculosis* using carbol fuchsin, acid decolorization, and methylene counter stain. [fig4.1(d)]

(a)

(b)



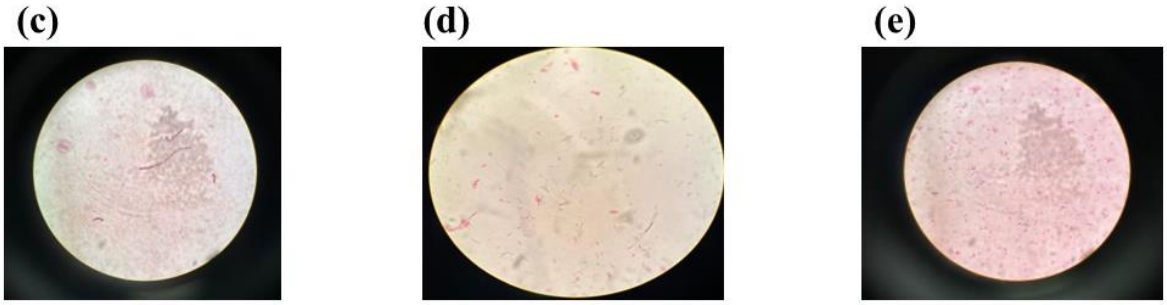
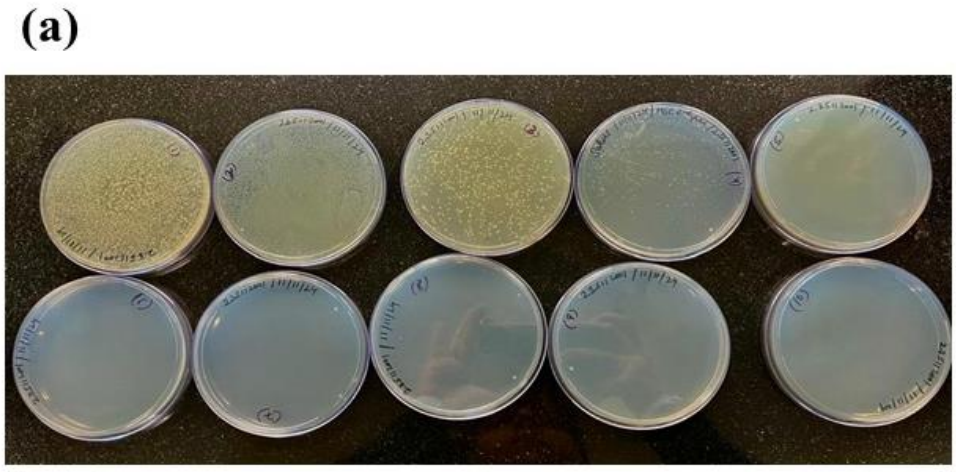


Fig.4.1: (a) Simple streaking, (b) Quadrant streaking of *E. coli* (c) Staining of Gram-positive (*Bacillus subtilis*) (d) Gram staining of gram-negative bacteria *(E. coli*) (e) AFB staining of *Mycobacterium smegmatis*

4.2 CFU count of *E. coli*

E. coli culture was taken from the JUIT campus and CFU count of *E. coli* was done to determine number of cells were present in the test tube. Serial; dilution of sample was made and 0.1ml of each dilution was plated in duplicate [Fig 4.2] shows representative plates obtained after incubation of plates.



(b)

Fig. 4.2: (a) Serial dilution of *E. coli* (10^{-1} to 10^{-10}) (b) countable colonies in 10^{-4}dillution

Formula to calculate CFU: -

CFU/mL = $\frac{47\times10^{-5}}{0.1}$ = 4.7 × 10^{-5} CFU/ml

4.3 Genomic DNA isolation from *E. coli*

Overnight grown *E. coli* was used to isolate its genomic DNA.and genomic DNA was run on 0.8%of agarose gel [Fig:4.3]

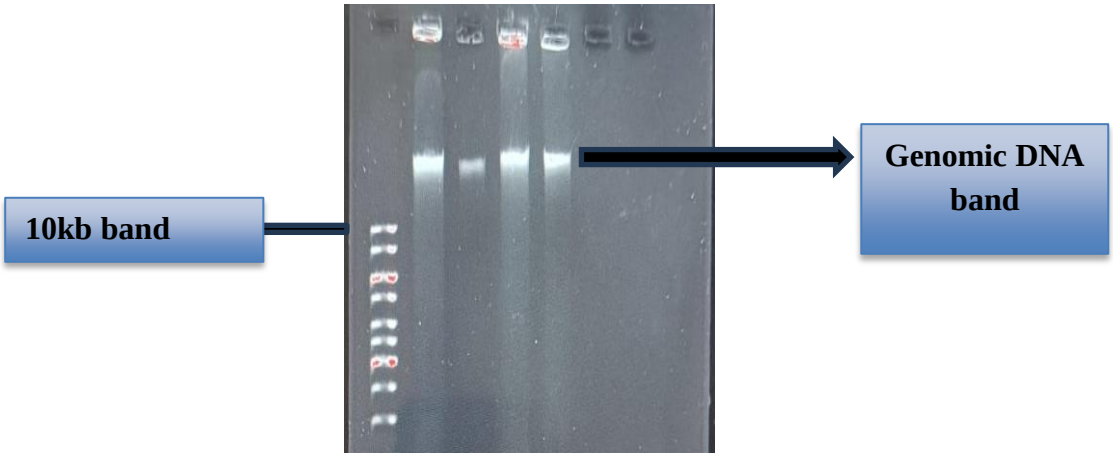


Fig 4.3: Genomic DNA extraction from *E. coli*

4.4 EXTRACTION

The process of plant extraction involves drying and grinding plant material, soaking it in a suitable solvent, filtering and evaporating the solvent to obtain the extract. [fig.4.4.1, and 4.5]

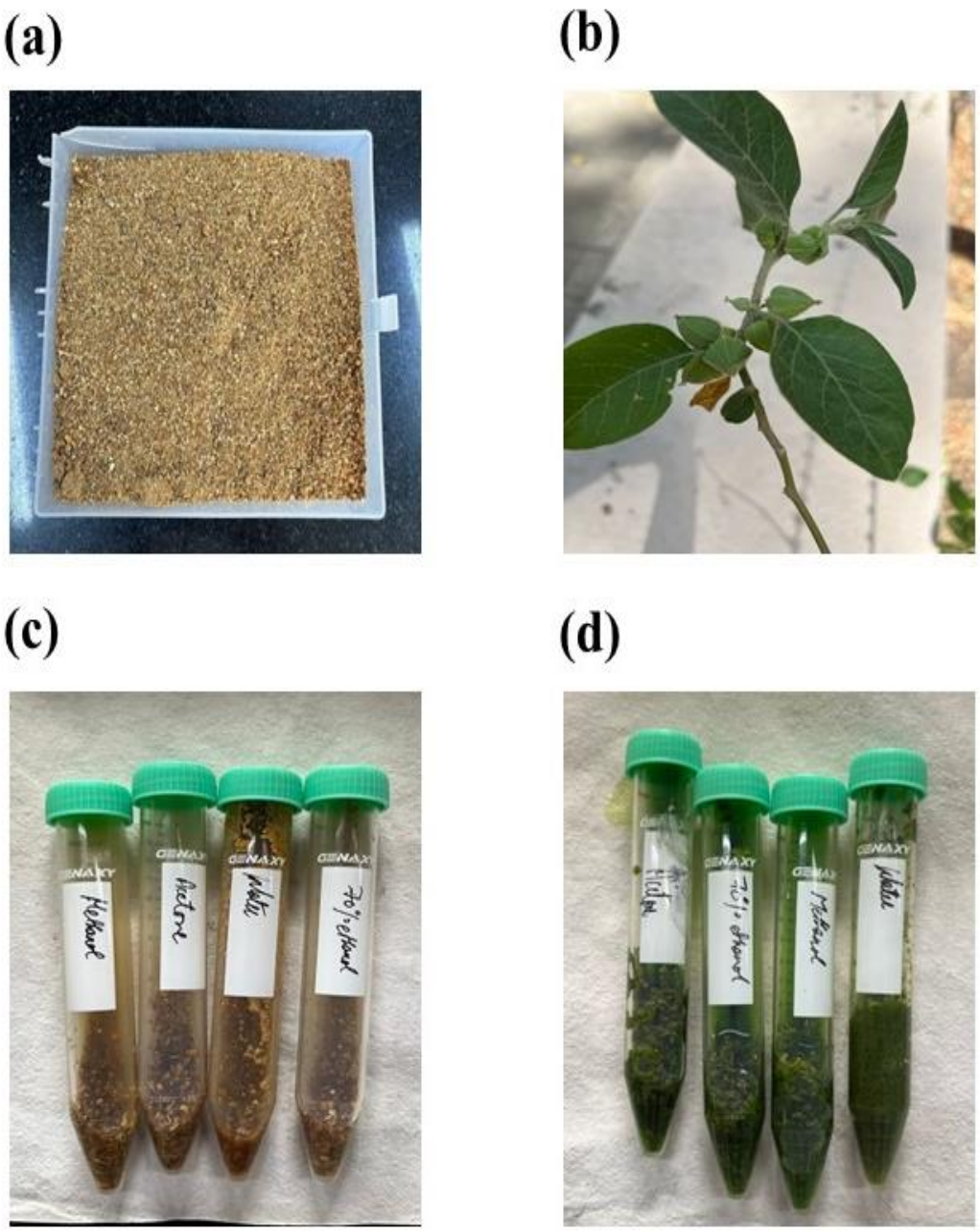


Fig. 4.4.1: (a) *Withania coagulans* fruit powder, (b) *Withania somnifera* fruit plant, (c,d) W*ithania coagulans* and *Withania somnifera* mixed in different solvents

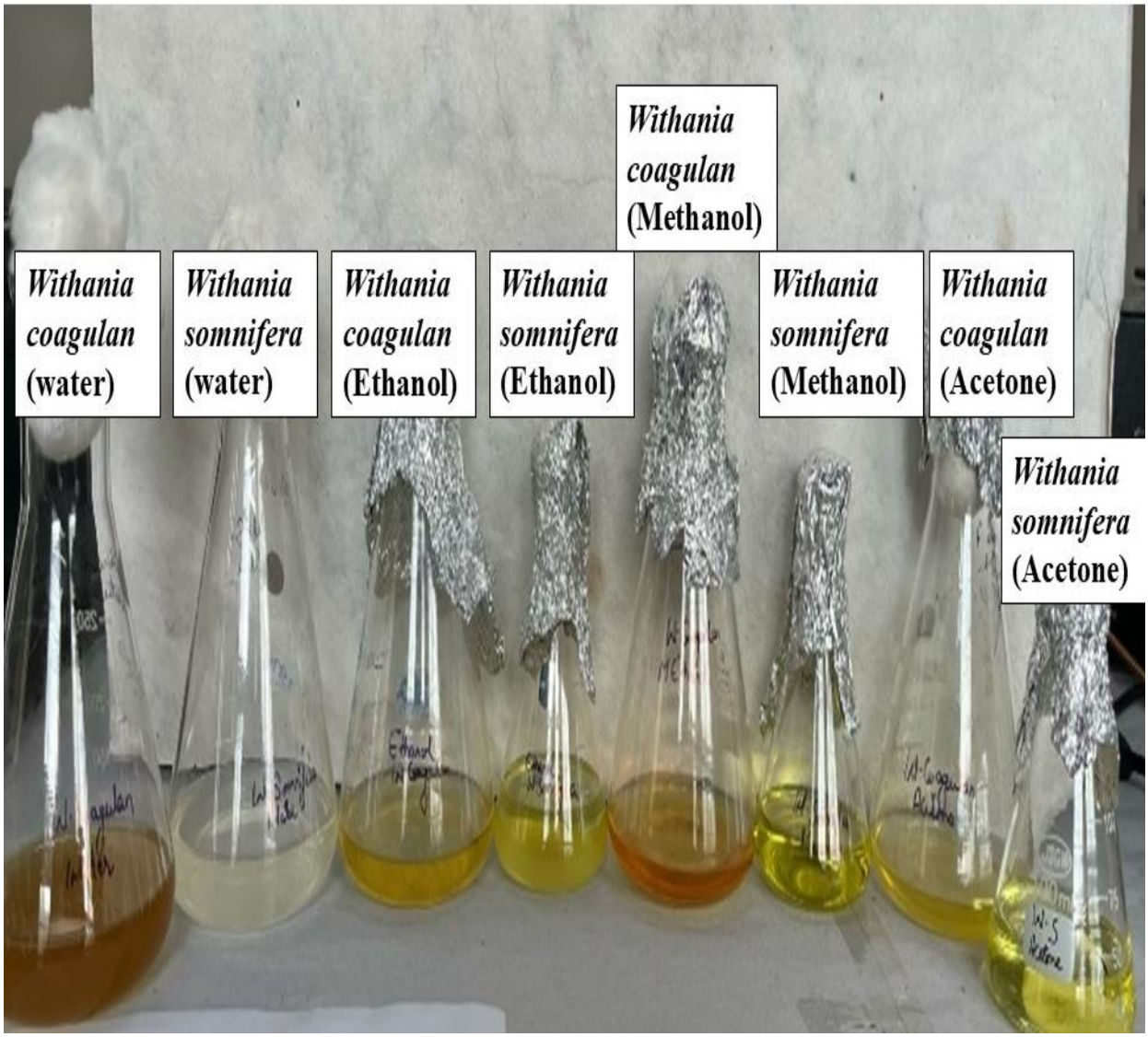


Fig. 4.5: Extract of W*ithania coagulans* and *Withania somnifera* mixed in different solvents

4.3 PHYTOCHEMICAL ANALYSIS

Table 4.3.1: Phytochemical analysis of *Withania somnifera* ethanol extract [36]

Phytochemical Test	Reagents Used	Procedure	Expected Result	Observation
Ferric Chloride Test (Tannins)	1 ml extract + 0.5 % ferric chloride	Mix in test tube	Blue-black tint	Negative
Salkowski Test (Glycosides)	1 ml extract + conc. H_2SO_4 + 2 ml chloroform	Add H_2SO_4 from side wall	Reddish-brown hue	Positive
Dragendorff's Test (Alkaloids)	1 ml extract + 2–3 drops Dragendorff's reagent	Mix in test tube	Turbidity/precipitate	Negative
Froth Test (Saponins)	2 ml extract + distilled water	Shake in test tube	Foam formation	Negative
Salkowski Test (Triterpenoids)	1 ml extract + conc. H_2SO_4 + 2 ml chloroform	Add H_2SO_4 along test tube wall	Reddish-brown hue near contact	Negative
Alkaline Reagent Test (Flavonoids)	2 ml extract + NaOH + HCl	Heat at 40°C for 5–10 min after adding HCl	Blue color appears	Negative
Molisch Test (Carbohydrates)	1 ml extract + 1–2 drops Molisch + H_2SO_4	Mix carefully	Violet/purple/blue ring	Positive

Ferric Chloride Test (Coumarins)	2 ml extract + ferric chloride + conc. HNO_3	Mix and observe	Green to yellow color	Negative
Borntrager's Test (Quinones)	2 ml extract + conc. H_2SO_4	Add along test tube wall	Red color appears	Positive
Ferric Chloride Test (Phenols)	1 ml extract + 1% ferric chloride + potassium ferrocyanide	Mix well	Greenish-blue tint	Negative

Table 4.3.2: Phytochemical analysis of *Withania coagulans* ethanol extract[37]

Test Performed	Reagents Used	Procedure Summary	Expected Observation	Test Result
Tannins (Ferric Chloride Test)	1 ml extract + 0.5% ferric chloride	Mixed in a test tube	Blue-black coloration	Negative
Glycosides (Salkowski Test)	1 ml extract + 2 ml conc. H_2SO_4 + 2 ml chloroform	Sulfuric acid added from the side of the test tube	Reddish-brown hue	Positive
Alkaloids (Dragendorff's) Test)	1 ml extract + 2–3 drops Dragendorff's reagent	Mixed in a test tube	Turbidity or precipitate formation	Positive
Saponins (Froth Test)	2 ml extract + distilled water	Shaken vigorously in a test tube	Persistent froth or foam formation	Positive

Triterpenoids (Salkowski Test)	1 ml extract + 2 ml conc. H_2SO_4 + 2 ml chloroform	Sulfuric acid added along the side of the test tube	Reddish-brown ring at interface	Positive
Flavonoids (Alkaline Reagent Test)	2 ml extract + NaOH followed by HCl	Heated at 40°C for 5–10 minutes after acid addition	Blue coloration appears	Positive
Carbohydrates (Molisch Test)	1 ml extract + 1–2 drops Molisch's reagent + conc. H_2SO_4	Mixed carefully in a test tube	Formation of violet, purple, or blue ring	Positive
Coumarins (Ferric Chloride Test)	2 ml extract + ferric chloride + conc. HNO_3	Mixed and observed for colour change	Green to yellow colour	Positive
Quinones (Borntrager's Test)	2 ml extract + conc. H_2SO_4	Sulfuric acid added along the side of the test tube	Appearance of red colour	Positive
Phenols (Ferric Chloride Test)	1 ml extract + 1% ferric chloride + potassium ferrocyanide	Mixed well and observed	Greenish-blue tint	Positive

Table 4.3.3: Photochemical analysis of *Withania coagulans* acetone extract [37]

Phytochemical Group	Test Performed	Reagents Used	Procedure Overview	Expected Colour Change or Reaction	Result
Tannins	Ferric Chloride Test	0.5% ferric chloride solution	The extract was mixed with ferric chloride in a test tube	Formation of a blue-black coloration	Negative
Glycosides	Salkowski Test	Concentrated H_2SO_4 and chloroform	H_2SO_4 was carefully added to the extract-chloroform mix along the test tube wall	Development of a reddish-brown ring	Positive
Alkaloids	Dragendorff's Test	Dragendorff's reagent	The reagent was added dropwise to the plant extract	Formation of a precipitate or turbidity	Positive
Saponins	Froth Test	Distilled water	The extract was shaken vigorously with distilled water	Persistent foam formation	Positive
Triterpenoids	Salkowski Test	Concentrated H_2SO_4 and chloroform	Acid was added gently along the test tube	Reddish-brown coloration	Positive

			containing the extract and chloroform	near the interface	
Flavonoids	Alkaline Reagent Test	Sodium hydroxide and hydrochloric acid	After mixing with NaOH and acidification, the mixture was gently heated	Appearance of a blue colour	Negative
Carbohydrates	Molisch Test	Molisch's reagent and concentrated H_2SO_4	A few drops of Molisch's reagent were added and mixed with H_2SO_4	Formation of a violet or purple ring	Negative
Coumarins	Ferric Chloride Test	Ferric chloride and concentrated HNO_3	The extract was mixed with both reagents and observed for colour change	Change from green to yellow	Negative
Quinones	Borntrager's Test	Concentrated H_2SO_4	Sulfuric acid was carefully added along the test tube wall	Development of a red coloration	Positive

Phenols	Ferric Chloride Test	Ferric chloride and potassium ferrocyanide	The mixture was stirred thoroughly and observed	Greenish-blue coloration	Negative

Table 4.3.4: Phytochemical analysis of *Withania somnifera* acetone extract

Phytochemical Group	Test Name	Reagents Used	Procedure Summary	Expected Observation	Result
Tannins	Ferric Chloride Test	1 mL extract + 0.5% ferric chloride	Mixed in a test tube	Blue-black coloration	Negative
Glycosides	Salkowski Test	1 mL extract + 2 mL conc. H_2SO_4 + 2 mL chloroform	H_2SO_4 added from the side of the test tube	Reddish-brown ring	Positive
Alkaloids	Dragendorff's Test	1 mL extract + 2–3 drops Dragendorff reagent	Reagents mixed directly in the test tube	Formation of turbidity or precipitate	Negative
Saponins	Froth Test	2 mL extract + distilled water	Solution shaken vigorously in the test tube	Persistent foam formation	Negative
Triterpenoids	Salkowski Test	1 mL extract + 2 mL conc. H_2SO_4 + 2 mL chloroform	Acid added carefully along the test tube wall	Reddish-brown coloration near the interface	Positive

Flavonoids	Alkaline Reagent Test	2 mL extract + NaOH + HCl	Color change observed after heating at 40°C for 5–10 minutes	Blue coloration appears	Negative
Carbohydrates	Molisch Test	1 mL extract + 1–2 drops Molisch reagent + conc. H_2SO_4	Reagents mixed carefully	Violet or purple ring formation	Positive
Coumarins	Ferric Chloride Test	2 mL extract + ferric chloride + conc. HNO_3	Mixed and observed for color change	Green to yellow coloration	Positive
Quinones	Borntrager's Test	2 mL extract + conc. H_2SO_4	Acid added along the side of the test tube	Red coloration appears	Positive
Phenols	Ferric Chloride Test	1 mL extract + 1% ferric chloride + potassium ferrocyanide	Mixed thoroughly	Greenish-blue tint	Negative
Proteins	Ninhydrin Test	1 mL extract + 0.2% ninhydrin solution	Reagents mixed in the test tube	Not performed	Negative

4.4 ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility test (AST) is used to determine the effectiveness of antibiotics against apecific bacteria by measuring zones of inhibition around then antibiotic discs on agar plate.

Fig.4.6 : No zone of inhibition is formed by 1:5 dillution of the *Wihania coagulan* extract

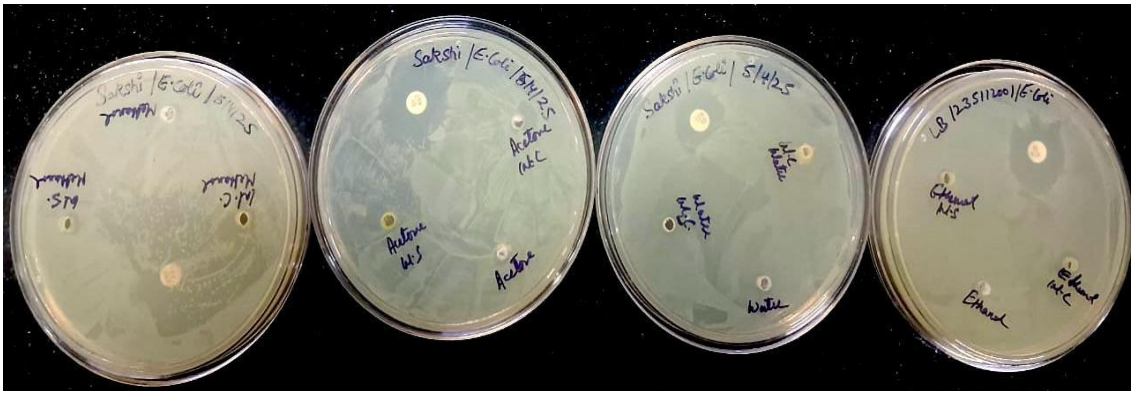


Fig.4.7 : No zone of inhibition is formed by *Wihania somnifera* extracts with the dillution 1:5

CHAPTER 5

CONCLUSION

CONCLUSION

Two of the most commonly employed medicinal plant of Ayurveda since centuries, *Withania coagulans* and *Withania somnifera*,are targeted in this reseach for antimicrobial activity. qualitative phytochemical screening for the presence of bioactive phytoconsituence such alkaloids, quinones, glucosides, triterpenoids and flavanoids etc.was established,espacially in ethanolic and acetone extract.these are also found to be effective antimicrobials for treating several infections.

The significant zone of inhibition against *E. coli* and *B. subtilis* was not produced by the antimicrobial senstivity test in the investigtion,which was carried out by utilizing the agar well diffusion method with 1:5 dillution of the extracts.This implies that in order to efficiently release the active ingredients,either the concentration was too low or the extraction condition could require more modification.

The phytochemical richness of both plant extracts supports their historic use and potential as sources of natural antibacterial agents, not with this limitation in direct antimicrobial outcomes.The results highlights the necessity of additional studies using larger extract concenteration, different extraction techniques,and wider range of microorganisms for screening, including resistant strains and fungii. Furthermore, understanding the distinct function and mechanism of each active chemical in microbial inhibitition may be aided by its purification and characterization.

In conclusion, Withania coagulans and Withania somnifera remain valuable candidates in the search of plant based antimicrobial agents, especially in the context of rising antibiotic resistance. Continues scientific exploration will be essential to unlock the full therapeutic potential.

REFERENCES

REFERENCES

[1]A. T. Mbaveng, F. Damen, İ. Çelik, P. Tane, V. Kuete, and T. Efferth, "Cytotoxicity of the crude extract and constituents of the bark of Fagara tessmannii towards multi-factorial drug resistant cancer cells," *J. Ethnopharmacol.*, vol. 235, pp. 28–37, 2019.

[2]M. K. Ahmad *et al.*, "Withania somnifera improves semen quality by regulating reproductive hormone levels and oxidative stress in seminal plasma of infertile males," *Fertil. Steril.*, vol. 94, no. 3, pp. 989–996, 2010.

[3]N. Singh, M. Bhalla, P. de Jager, and M. Gilca, "An overview on ashwagandha: a Rasayana (rejuvenator) of Ayurveda," *Afr. J. Tradit. Complement. Altern. Med.*, vol. 8, no. 5S, 2011, Accessed: Jun. 25, 2025. [Online]. Available: https://www.ajol.info/index.php/ajtcam/article/view/67963

[4]T. J. Morano, N. J. Bailey, C. M. Cahill, and É. C. Dumont, "Nuclei-and condition-specific responses to pain in the bed nucleus of the stria terminalis," *Prog. Neuropsychopharmacol. Biol. Psychiatry*, vol. 32, no. 3, pp. 643–650, 2008.

[5]N. Tandon and S. S. Yadav, "Contributions of Indian Council of Medical Research (ICMR) in the area of Medicinal plants/Traditional medicine," *J. Ethnopharmacol.*, vol. 197, pp. 39–45, 2017.

[6]K. Chandrasekhar, J. Kapoor, and S. Anishetty, "A Prospective, Randomized Double-Blind, Placebo-Controlled Study of Safety and Efficacy of a High-Concentration Full-Spectrum Extract of Ashwagandha Root in Reducing Stress and Anxiety in Adults," *Indian J. Psychol. Med.*, vol. 34, no. 3, pp. 255–262, Jul. 2012, doi: 10.4103/0253-7176.106022.

[7]N. J. Dar, A. Hamid, and M. Ahmad, “Pharmacologic overview of Withania somnifera, the Indian Ginseng,” *Cell. Mol. Life Sci.*, vol. 72, no. 23, pp. 4445–4460, Dec. 2015, doi: 10.1007/s00018-015-2012-1.

[8]M. H. Mirjalili, E. Moyano, M. Bonfill, R. M. Cusido, and J. Palazón, “Steroidal lactones from Withania somnifera, an ancient plant for novel medicine,” *Molecules*, vol. 14, no. 7, pp. 2373–2393, 2009.

[9]P. Sinoriya, R. Kaushik, A. Sinoria, and P. K. Gaur, “Comprehensive Review on Withania coagulans Dunal: Unveiling Pharmacognosy, Phytochemistry and Pharmacological Potentials.,” *Pharmacogn. Rev.*, vol. 18, no. 35, 2024, Accessed: Jun. 25, 2025. [Online]. Available: https://phcogrev.com/sites/default/files/PharmacognRev-18-35-47.pdf

[10] R. Maurya, “Chemistry and pharmacology of Withania coagulans: an Ayurvedic remedy,” *J. Pharm. Pharmacol.*, vol. 62, no. 2, pp. 153–160, 2010.

[11] M. H. Mirjalili, E. Moyano, M. Bonfill, R. M. Cusido, and J. Palazón, “Steroidal lactones from Withania somnifera, an ancient plant for novel medicine,” *Molecules*, vol. 14, no. 7, pp. 2373–2393, 2009.

[12] N. J. Dar, A. Hamid, and M. Ahmad, “Pharmacologic overview of Withania somnifera, the Indian Ginseng,” *Cell. Mol. Life Sci.*, vol. 72, no. 23, pp. 4445–4460, Dec. 2015, doi: 10.1007/s00018-015-2012-1.

[13] N. J. Dar, A. Hamid, and M. Ahmad, “Pharmacologic overview of Withania somnifera, the Indian Ginseng,” *Cell. Mol. Life Sci.*,

vol. 72, no. 23, pp. 4445–4460, Dec. 2015, doi: 10.1007/s00018-015-2012-1.

[14] R. Jain, S. Kachhwaha, and S. L. Kothari, "Phytochemistry, pharmacology, and biotechnology of Withania somnifera and Withania coagulans: A review," *J. Med. Plants Res.*, vol. 6, no. 41, pp. 5388–5399, 2012.

[15] R. Jain, S. Kachhwaha, and S. L. Kothari, "Phytochemistry, pharmacology, and biotechnology of Withania somnifera and Withania coagulans: A review," *J. Med. Plants Res.*, vol. 6, no. 41, pp. 5388–5399, 2012.

[16] K. O. Soetan, T. O. Ajibade, and A. S. Akinrinde, "Saponins; a ubiquitous phytochemical: a review of its biochemical, physiological and pharmacological effects," *Recent Prog Med Plants*, vol. 43, pp. 1–24, 2014.

[17] C. Baskaran and S. Velu, "Phytochemical analysis and in-vitro antimicrobial activity of Withania somnifera (Ashwagandha)," *J Nat Prod Plant Resour*, vol. 2, no. 6, pp. 711–6, 2012.

[18] K. B. Pandey and S. I. Rizvi, "Plant Polyphenols as Dietary Antioxidants in Human Health and Disease," *Oxid. Med. Cell. Longev.*, vol. 2, no. 5, pp. 270–278, Jan. 2009, doi: 10.4161/oxim.2.5.9498.

[19] M. F. Azhar *et al.*, "Antioxidant and phytochemical composition of leaves, stem and root extracts of Withania coagulans and Withania somnifera," *Z. Arznei-Gewurzpflanzen*, vol. 25, pp. 27–30, 2020.

[20] R. Murugan *et al.*, "Withaferin A targets the membrane of Pseudomonas aeruginosa and mitigates the inflammation in zebrafish larvae; an in vitro and in vivo approach," *Microb. Pathog.*, vol. 172, p. 105778, 2022.

[21] R. Jan, M. Khan, S. Asaf, Lubna, S. Asif, and K.-M. Kim, "Bioactivity and therapeutic potential of kaempferol and quercetin: new insights for plant and human health," *Plants*, vol. 11, no. 19, p. 2623, 2022.

[22] D. Ming *et al.*, "Kaempferol inhibits the primary attachment phase of biofilm formation in Staphylococcus aureus," *Front. Microbiol.*, vol. 8, p. 2263, 2017.

[23] D. Sebaro, K. S. Moorthy, A. Tora, and M. Senapathy, "In-vitro antibacterial activity of crude leaf extracts of Withania somnifera and Moringa oleifera against some selected bacterial pathogens," *Med. Plants-Int. J. Phytomedicines Relat. Ind.*, vol. 15, no. 4, pp. 784–793, 2023.

[24] M. Kuśmierska, J. Kuśmierski, and O. Kwaśniewska, "Exploring the therapeutic potential of Ashwagandha (Withania somnifera) supplementation in alleviating stress and stress-related disorders," *Qual. Sport*, vol. 15, pp. 51854–51854, 2024.

[25] A. Samad, N. N. Rajpoot, H. Ayaz, and N. Sadiq, "Effect of Withania coagulans and liraglutide on serum Glp-1, postprandial and fasting blood glucose in streptozotocin induced diabetic rats," *J. Bahria Univ. Med. Dent. Coll.*, vol. 9, no. 2, pp. 120–123, 2019.

[26] R. Murugan *et al.*, "Withaferin A targets the membrane of Pseudomonas aeruginosa and mitigates the inflammation in zebrafish larvae; an in vitro and in vivo approach," *Microb. Pathog.*, vol. 172, p. 105778, 2022.

[27] R. Jan, M. Khan, S. Asaf, Lubna, S. Asif, and K.-M. Kim, "Bioactivity and therapeutic potential of kaempferol and quercetin: new insights for plant and human health," *Plants*, vol. 11, no. 19, p. 2623, 2022.

[28] D. Ming *et al.*, “Kaempferol inhibits the primary attachment phase of biofilm formation in Staphylococcus aureus,” *Front. Microbiol.*, vol. 8, p. 2263, 2017.

[29] N. J. Dar, A. Hamid, and M. Ahmad, “Pharmacologic overview of Withania somnifera, the Indian Ginseng,” *Cell. Mol. Life Sci.*, vol. 72, no. 23, pp. 4445–4460, Dec. 2015, doi: 10.1007/s00018-015-2012-1.

[30] K. Lucius, “Clinical Evidence for the Use of Ashwagandha, *Withania somnifera*,” *Integr. Complement. Ther.*, vol. 31, no. 2, pp. 54–62, Apr. 2025, doi: 10.1089/ict.2025.28106.kl.

[31] A. Samad, N. N. Rajpoot, H. Ayaz, and N. Sadiq, “Effect of Withania coagulans and liraglutide on serum Glp-1, postprandial and fasting blood glucose in streptozotocin induced diabetic rats,” *J. Bahria Univ. Med. Dent. Coll.*, vol. 9, no. 2, pp. 120–123, 2019.

[32] G. Visweswari, R. Christopher, and W. Rajendra, “Phytochemical screening of active secondary metabolites present in Withania somnifera root: role in traditional medicine,” *Int. J. Pharm. Sci. Res.*, vol. 4, no. 7, p. 2770, 2013.

[33] D. Jaiswal, P. K. Rai, and G. Watal, “Antidiabetic effect of Withania coagulans in experimental rats,” *Indian J. Clin. Biochem.*, vol. 24, no. 1, pp. 88–93, Jan. 2009, doi: 10.1007/s12291-009-0015-0.

[34] K. Sampathkumar, S. Riyajan, C. K. Tan, P. Demokritou, N. Chudapongse, and S. C. J. Loo, “Small-Intestine-Specific Delivery of Antidiabetic Extracts from *Withania coagulans* Using Polysaccharide-Based Enteric-Coated Nanoparticles,” *ACS Omega*, vol. 4, no. 7, pp. 12049–12057, Jul. 2019, doi: 10.1021/acsomega.9b00823.

[35] R. Murugan *et al.*, “Withaferin A targets the membrane of Pseudomonas aeruginosa and mitigates the inflammation in zebrafish larvae; an in vitro and in vivo approach,” *Microb. Pathog.*, vol. 172, p. 105778, 2022.

[36] M. Kuśmierska, J. Kuśmierski, and O. Kwaśniewska, “Exploring the therapeutic potential of Ashwagandha (Withania somnifera) supplementation in alleviating stress and stress-related disorders,” *Qual. Sport*, vol. 15, pp. 51854–51854, 2024.

[37] M. C. Egbujor, S. A. Egu, V. I. Okonkwo, A. D. Jacob, P. I. Egwuatu, and I. S. Amasiatu, “Antioxidant drug design: historical and recent developments,” *J Pharm Res Int*, vol. 32, no. 41, pp. 36–56, 2021.

[38] M. F. Anjum *et al.*, “The potential of using E. coli as an indicator for the surveillance of antimicrobial resistance (AMR) in the environment,” *Curr. Opin. Microbiol.*, vol. 64, pp. 152–158, 2021.

[39] P. Bisht and V. Rawat, “Antibacterial activity of Withania somnifera against Gram-positive isolates from pus samples,” *AYU Int. Q. J. Res. Ayurveda*, vol. 35, no. 3, pp. 330–332, 2014.