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EVALUATION OF *INVITRO* ANTIMICROBIAL ACTIVITY AND CYTOTOXICITY POTENTIAL OF AQUEOUS ROOT EXTRACT OF *SMILAX CHINA* LINN'

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ABSTRACT

Traditional Korean medicine has long utilized the root of Smilax china L., and our research has unveiled its remarkable antimicrobial and cytotoxic potential. This study was designed to assess the antimicrobial capabilities and cytotoxicity of an aqueous root extract from Smilax china L. against Staphylococcus aureus and Pseudomonas aeruginosa, while also exploring itseffectiveness in inhibiting biofilm formation. To evaluate the antimicrobial activity, we employed the agar well diffusion method with varying concentrations (25, 50, 100, 200, 400 µg/mL) of the root extract. These results were compared to the standard antibiotics chloramphenicol (30 mcg/disc) and Ciprofloxacin (5 mcg/disc). The Minimum Inhibitory Concentration (MIC) for the Smilax china root extract against S. aureus was found to be 800 µg/mL, resulting in a 50% inhibition compared to the control. The Minimum Bactericidal Concentration (MBC) was subsequently determined to be 1600 µg/mL. For P. aeruginosa, the MIC was established at 400 µg/mL, with a 50% inhibition compared to the control, and the MBC was determined to be 800 µg/mL. Notably, the root extract demonstrated biofilm inhibition potential, with biofilms being less dense at a concentration of 1600 µg/mL for S. aureus and 800 µg/mL for P. aeruginosa. Cytotoxicity assessments on RAW 264.7 mouse macrophage cells, using the MTT assay, revealed that the Smilax china root extract inhibited cell growth even at very low concentrations. The IC50 was calculated to be 3.1 µg/mL, resulting in a 50.6% inhibition rate. These findings underscore the promising antimicrobial and cytotoxic properties of the Smilax china root extract, which may hold significant therapeutic potential.

KEYWORDS: Smilax china L., *Staphylococcus aureu, Pseudomonas aeruginosa*, RAW 264.7 cells, Antimicrobial activity, Cytotoxic potential acitivity.

1. INTRODUCTION

Nature has long been a valuable source of medicinal remedies, aiding in human health maintenance since ancient times. The world is blessed with an abundant array of medicinal plants, without which human survival on Earth would be precarious. These plants and their derivatives play a vital role in various medical systems like Chinese medicine, Ayurveda, Siddha, Unani, and Tibetan medicine. Ancient texts such as the Rigveda, Yajurveda, Atharvaveda, Charak Samhita, and Sushrut Samhita document the use of plants to address a wide range of health issues. Plants produce a diverse range of bioactive molecules, making them a rich source of various types of medicines. Natural remedies derived from plants, often referred to as "green medicines," are generally safer and healthier than synthetic counterparts. Numerous herbal medicines effectively manage various diseases, offering minimal toxicity, cost-effectiveness, pharmacological activity, and straightforward solutions for many human ailments, as compared to synthetic drugs that are susceptible to adulteration and side effects.

Medicinal plants serve as a crucial reservoir of novel chemical compounds with potential therapeutic applications, particularly in the treatment of chronic and infectious diseases. Plant-derived natural products can serve as templates for new drug development and possess fascinating biological activities, including anti-diabetic, antioxidant, antibacterial, anti-inflammatory, antipyretic, and gastroprotective effects. Every part of a plant can be utilized for herbal medicine, whether it be the leaves, stems, flowers, bark, fruits, peels, rhizomes, essential oils, latex, or buds. The medicinal value of plants primarily resides in the phytochemicals they contain, which exert specific physiological actions on the human body. Plants synthesize and accumulate various phytochemicals such as tannins, flavonoids, phenolic compounds, glycosides, steroids, and saponins, many of which are potent bioactive compounds suitable for the development of useful drugs. These phytochemicals play a pivotal role in regulating, protecting against, and controlling numerous diseases, although their active principles may vary across different plant species due to their diverse biochemical nature.

The global concern surrounding bacterial infections centers on the development and spread of resistance to existing antibiotics. An alarming aspect of treating bacterial infections is bacteria's ability to acquire resistance to antimicrobial agents. The indiscriminate use of antibiotics appears to fuel the emergence of pathogens resistant to multiple drugs. Multiple

drug resistance not only heightens morbidity and mortality rates but also increases healthcare expenditures for patient management and the implementation of infection control measures. It is imperative to continuously search for new antimicrobial compounds with diverse chemical structures and novel mechanisms of action. As a result, measures must be taken to regulate antibiotic usage, gain a deeper understanding of the genetic mechanisms behind resistance, and persist in the development of new drugs. In contemporary medical practice, it is common to employ a combination of two or more antibiotics with distinct modes of action to hinder the expansion of antibiotic resistance and enhance treatment outcomes. The synergy between antimicrobial agents may result from complex interactions that prove more effective in inhibiting microorganisms when compared to individual agents. Diverse approaches are employed to combat infections caused by multidrug-resistant bacterial strains, one of which involves the utilization of phytochemicals from plants with antimicrobial properties to curb the spread of infections.

Another alternative strategy is to devise novel synergistic combinations that incorporate commercially available antibiotics with or without plant-derived phytochemicals possessing antimicrobial properties. Such combination therapy has the potential to reduce the minimum effective dose of antibiotics required for treatment and mitigate side effects. Plant crude extracts consist of a blend of compounds that can enhance the efficacy of various antibiotics. Several in vitro studies have documented the use of plant extracts in conjunction with antibiotics to combat resistant strains effectively.

Table 1: Therapeutic uses of herbals.

Plant name	Family	Therapeutic uses	Microorganisms
Manilkara zapoto L.	Sapotaceae	Diuretic, tonic, febrifuge, antibiotics, astringent and febrifuge	*Bacillus subtulis, Staphylococcus aureus, pseudomonas aeruginosa, Escherichia coli.*
Psidium guajava L.	Myrtaceae	Astringent, haemostatic, constipating, antiemetic, febrifuge, antispasmodic, tonic, laxative	*Staphylococcus aureus, pseudomonas aeruginosa, Escherichia coli, Bacillus subtulis.*
Punica granatum L.	Myrtaceae	Astringent, anthelmintic, tonic, aphrodisiac, laxative, diuretic, cardio tonic, vomiting	*pseudomonas aeruginosa, Escherichia coli.*

1.1. CYTOTOXICITY

Cytotoxicity refers to the harmful effects caused by the interaction of chemotherapy drugs with living cells. In the context of nanoparticles, cytotoxicity tests play a crucial role in assessing their potential biomedical applications. These tests are essential for evaluating the suitability of nanoparticles for various medical uses. To determine cytotoxicity and cell viability, different dyes like Trypan Blue, Alamar Blue, neutral red, and Coomassie Blue are employed. This method distinguishes between various types of cells based on the colors they exhibit. Cells are categorized by analyzing the ratio of color uptake in both living and dead cells. Other techniques used for assessing cytotoxicity include the tritium-labeled thymidine uptake assay, the MTT method, WST assay, and assays based on dehydrogenase activity (Li et al., 2012).

The capacity of specific substances or mediator cells to eliminate viable cells can result from various factors. These factors include exposure to chemicals, such as drugs or cosmetics, interaction with other cell types like NK and T cells, or exposure to adverse physical or environmental conditions such as radiation, extreme temperatures, or pressure.

Cytotoxicity can result in three potential outcomes for healthy living cells

1. Necrosis, which is characterized by the rapid loss of membrane integrity and subsequent cell lysis, leading to accidental cell death.
2. Apoptosis, a more deliberate and genetically regulated form of cell death that occurs gradually and in an orderly manner.
3. Cytostasis, where a cell remains alive but experiences a reduction in its ability to actively grow and divide.

1.2. Significance of Cytotoxicity Assessment

Cytotoxicity measurement holds substantial importance for two primary reasons.

First, it aids in identifying specific cells that need to be eliminated, which is vital in fields like cancer treatment and immunotherapy.

Second, it helps ensure the safety of cells by detecting and excluding potential harm from chemicals and drugs.

1.3. Applications

The assessment of cytotoxicity finds applications in various domains, including:

1. Drug Discovery: It plays a crucial role in the drug development process, allowing researchers to identify compounds or conditions that affect cell viability.
2. Oncology Research: Cytotoxicity assessment is pivotal in oncology studies to understand how treatments impact cancer cells.
3. Safety Evaluation: It is used to evaluate the safety of substances such as pesticides, plant extracts, food additives, cosmetics, and industrial chemicals to ensure they do not harm cells.

1.4. Advantages of In vitro Cytotoxicity Studies

Conducting cytotoxicity studies in vitro offers several advantages, including:

1. Speed: In vitro assays provide rapid results, expediting research and decision-making.
2. Reduced Cost: They are cost-effective compared to in vivo studies involving animals or other models.
3. Potential for Automation: In vitro assays can be automated, enhancing efficiency and consistency.
4. Enhanced Prediction: Testing on specific cell lines improves the predictive value of results.
5. No Need for Testing Models: In vitro studies eliminate the need for complex testing models like plants or animals.
6. Scalability: They are suitable for testing a large number of samples simultaneously.

1.5. Cytotoxicity Measurement Methods

To assess cytotoxicity, researchers rely on certain criteria indicating cell viability and functionality. These criteria include:

1. Metabolism: Viable cells should demonstrate metabolic activity.
2. Proliferation: They should have the ability to divide and multiply.
3. Membrane Integrity: Viable cells maintain intact cell membrane integrity.

Based on these criteria, various assays have been developed to measure cytotoxicity and cell viability, including those that assess:

- Cell membrane permeability
- Enzyme activity
- Cell adherence
- ATP production

- Coenzyme production
- Nucleotide uptake activity

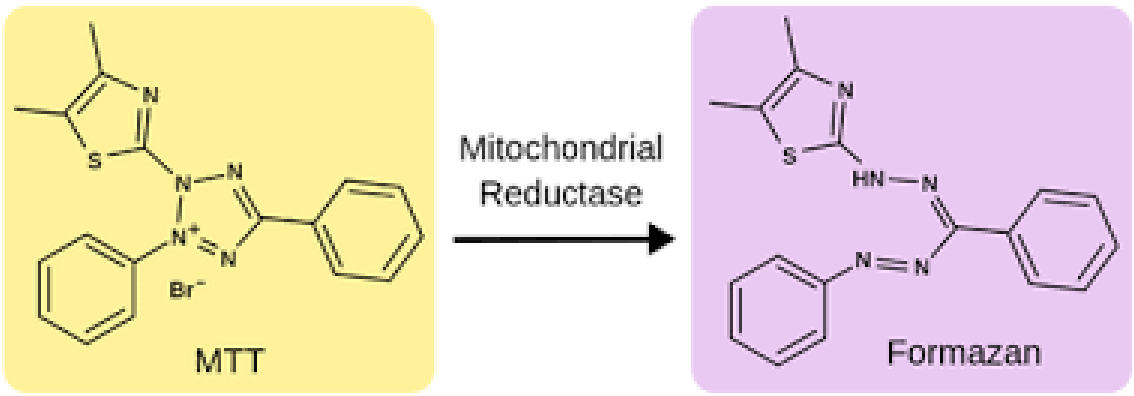
1.6. Cytotoxicity Assays

To ascertain cell death resulting from various mechanisms, cytotoxicity and cell viability assays are essential. These assays are characterized by their affordability, reliability, and reproducibility. They can be categorized based on the types of endpoints measured, such as color changes, fluorescence, or luminescence.

1. Dye Exclusion Assays: These include Trypan blue, eosin, Congo red, erythrosine B assays.
2. Colorimetric Assays: Examples are the MTT assay, XTT assay, WST-1 assay, NST-8 assay, LDH assay, SRB assay, NRV assay, and crystal violet assay.
3. Fluorimetric Assays: Notable examples comprise the Alamar blue assay and CFDA-AM assay.
4. Luminometric Assays: These encompass the ATP assay and real-time viability assay.

1.7. MTT ASSAY

Principle



MTT, after methyl-thiazolyl-tetrazolium (Mosmann, 1983) are widely used for assessment of cytotoxicity, cell viability, and proliferation studies in cell biology (Horobin and Kiernan, 2002, Berridge et al., 2005, Van Meerloo et al., 2011).

Water soluble yellow MTT reduced to purple insoluble formazan by mitochondrial dehydrogenase.

Water insoluble formazan can be solubilized using isopropanol or other solvents.

The dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye.

Non viable cells will not produce dehydrogenase.

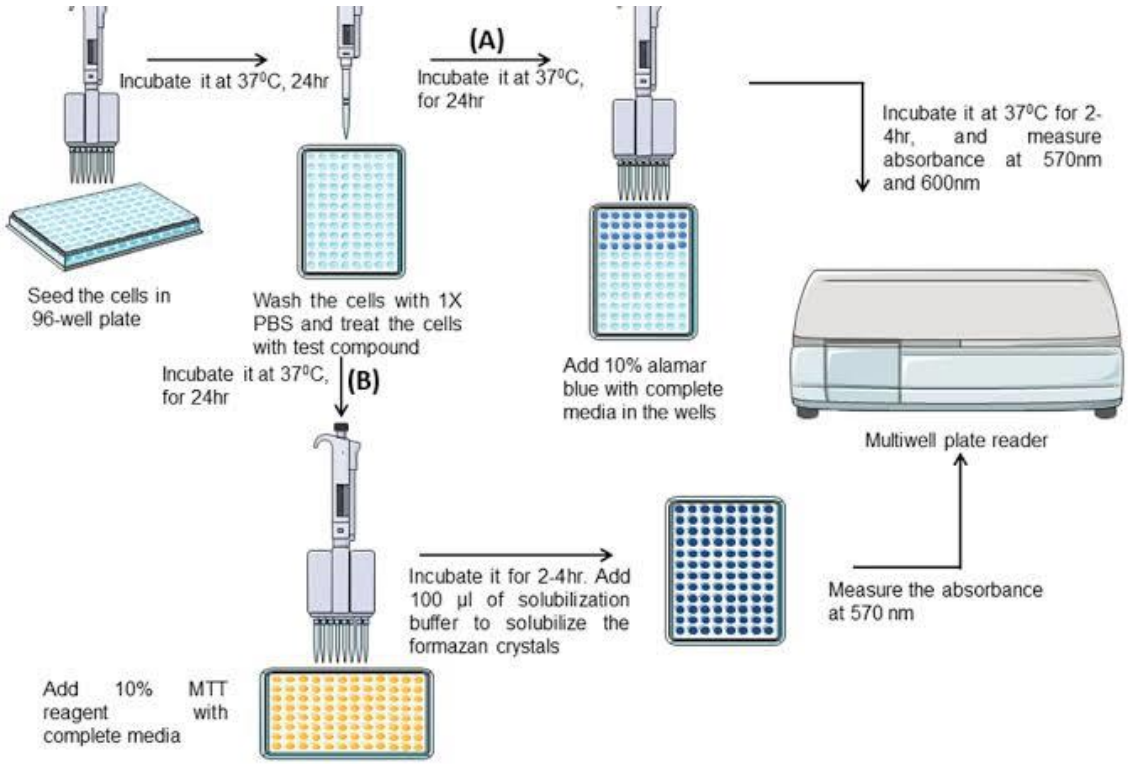
Thus dead cells do not cause this change. The amount of formazan produce is directly proportional to the number of viable cells in the sample.

Reagent preparation

MTT solution – 5mg/ml MTT in PBS solution must be filter sterilized after adding MTT.
For long time storage keep the MTT solution in -20 degree Celsius.

MTT solvent
Option – 1: 400 ul HCL 1 M, 10 ml trion 100%, 90ml isopropanol.
Option – 2: DMSO 100% room temperature.

PROCEDURE



MTT assay procedure

MTT gives a yellowish aqueous solution which, on reduction by dehydrogenases and reducing agents present in metabolically active cells, yields a water insoluble violet-blue

formazan (Fig. 1A). The lipid soluble formazan product may be extracted with organic solvents and estimated by spectrophotometry. It is currently widely thought that the amount of MTT formazan is directly proportional to the number of living cells (Van Meerloo et al., 2011), however such conclusions have been seriously questioned (Etxeberria et al., 2011).

Ever since early applications of the method as a viability assay for cultured cells (Mosmann, 1983), MTT has been considered to be reduced by the activity of mitochondrial dehydrogenases in living cells. Consequently it has been assumed that the sites of reduction, and of formation of the formazan precipitate, were the mitochondria. In particular it has been claimed that the mitochondrial succinate dehydrogenase of viable cells reduced MTT to the corresponding formazan (Saravanan et al., 2003).

Advantages

- No transfer of the cells, the entire assay is performed in a single micro plate.
- MTT is metabolized by all cells, the assay can be used with all cell types.
- Inexpensive.

Disadvantage

- Assay is not linear over a broad logarithmic cell proliferation range due to the ELISA plate reader.
- Insoluble reaction product resolubilization of the reaction product required.
- Cannot take multiple time points in a Sample assay.
- Cells with low metabolic activity must be used in high numbers.

2. AIM AND OBJECTIVE

Based on the literature review and considering that there are very few studies investigating the antimicrobial potential of *S. china*, the present study aimed to evaluate the antimicrobial activity and cytotoxicity potential of aqueous root extract of *Smilax china* L. Specific objectives are listed below:

- To identify the antimicrobial activity of aqueous root extract of *Smilax china* L. against *Staphylococcus aureus* and *Pseudomonas aeruginosa* by agar well diffusion method and further estimate the Minimum Inhibitory Concentration (MIC) and Maximum Bactericidal Concentration (MBC).
- To evaluate the cytotoxicity potential of the extract of *Smilax China.*

- To evaluate the biofilm inhibition potential of the extract of *Smilax china*.

3. PLANT PROFILE

Smilax china L., a member of the Smilacaceae family, boasts a global presence, thriving in tropical and temperate regions, with a particularly robust presence in East Asia (Khan et al., 2009; Seo et al., 2012). This esteemed medicinal plant, known as Smilax China or China root in English, holds immense value in traditional Ayurvedic and Chinese medicinal practices. Chopchini, as it is called, goes by different names in various languages. In Hindi, it's referred to as Chopchini, Chobchini, or Toupchini, while Tamil speakers know it as Ayadi, Malayalam as Kaltamara, Marathi as Ghotvel, Telugu as Kondadantena, Kannada as Kaadu hambu, Bengali as Kumariak, and Oriya as Mootrilata.

3.1. SCIENTIFIC CLASSIFICATION

Kingdom	:	Plantae
Clade	:	Tracheophytes
Clade	:	Angiosperms
Order	:	Lilliales
Family	:	Smilacaceae
Genus	:	Smilax
Species	:	*S. China*

BINOMIAL NAME

***Smilax china* L**

Chopchini is an evergreen and deciduous shrub that grows about 10 m in height. The leaves of this plant are heart-shaped, ranging in size from 5-30 cm in different species. Flowers join to form a white cluster and green in hue, and the flowering season is from May to June. The plant produces round berries that are red to blue-black, and the diameter of the fruit is 5-10 m. The fruit is rubbery in texture with a single ample seed inside. *Smilax china* is a native plant of China, Korea, Taiwan, and Japan. It is widely distributed in forests, hillsides, and shaded areas along the valleys in India, Vietnam, the Philippines, and Myanmar.

Figure 1: (a) Leaves of *S. china* (b) root chunks of *S. china.*

This plant is a perennial and somewhat woody climber with aculeated skin and paired tendrils that aid in climbing. Several studies have shown that the tubers of *Smilax china* L. have been used in traditional medicine for the treatment of furunculosis, gout, tumors, and inflammation (Chen et al, 2011; Huang et al, 2007; Li et al, 2007; Shuan et al, 2006; Ruan, 2005). The rhizomes or roots of this wonder herb possess analgesic, anti-inflammatory, diuretic, stimulant, carminative, laxative, and tonic properties. Further, it is also highly valued for treating several venereal disorders and skin-related woes.

3.2. ACTIVE CONSTITUENTS

The main active components of *Smilax china L.* are stilbenes, flavonoids, polyphenols and steroidal saponins (Ruan et al, 2002, Xu et al, 2008). Polyphenol compounds like resveratrol are also found in *Smilax china* L.

Smilax china L. is an important herb used in traditional Chinese medicine with various pharmacological properties, and is also consumed as food in certain parts of China. Several medicinal properties of *Smilax china* L. have been studied including antioxidant, anti-inflammatory, anti-obesity and anti-cancer effects. *Smilax china* root, which is rich in resveratrol and oxyresveratrol, has been used as emergency foods as well as folk medicine.

Lincha et al (2016) We isolated 10 compounds from ethyl acetate (EtOAc) fraction of 70% EtOH extract of SCE and investigated their inhibitory effect on nicotine-induced ED in endothelial cells. Kaempferol, kaempferol 7-O-α-L-rhamnopyranoside, puerarin and ferulic acid showed strong inhibition of nicotine-induced vascular cell adhesion molecule (VCAM-

1) expression while kaempferol, kaempferin, and caffeic acid attenuated intercellular adhesion molecule (ICAM-1) expression. Lepidoside, caffeic acid and methylsuccinic acid caused the highest up-regulated expression of endothelial nitric oxide synthase at the protein level with caffeic acid and ferulic acid showing strong inhibitory effects on inducible nitric oxide synthase (iNOS) expression. In addition, ferulic acid and kaempferol showed inhibition against interleukin-8 (IL-8) and interleukin-1β (IL-1β) expression while ferulic acid and caffeic acid showed comparatively higher inhibition of ED associated tumor necrosis factor-α (TNF-α) expression.

Xu et al (2022) reported that eighteen polyphenolic compounds were identified by LC-MS/MS analysis. The methanolic extract (MSC) of SC was subjected to fractionation using different solvents including n-hexane, benzene, chloroform, ethyl acetate and n-butanol. Results revealed that ethyl acetate fraction showed maximum phenolic and flavonoid, urease, tyrosinase and Butyrylcholinesterase.

Smilax china L. root extract (SSCR) containing chlorogenic acid. Chlorogenic acid is an identified bioactive component in SSCR by ultra performance liquid chromatography/photodiode array/electro spray ionization/mass spectroscopy (UPLC/PDA/ESI/MS).

Considering the multitude of biological effects that this plant possesses, the present study was designed to investigate the antimicrobial, cytotoxicity and biofilm prevention of the aqueous root extract.

4. MATERIAL AND METHODS

4.1. COLLECTION OF ROOT POWDER

Root powder of *Smilax china* was obtained commercially. Chopchini root powder (*smilax china*) 7 Oz (200 g) shop by https://amzn.eu/d/b96yMzR www.bixabotanical.com manufactured by plot No: 101, sector-1, pithampur, Dist, Dhar (M.P) – 454 775 India.

4.2. EXTRACT PREPARATION

The *smilax china* root powder was taken (250g) and 1000 mL of H_2O was added. The content was kept for stirring at 60 °C for 72 hrs with occasionally shaking. Then, the aqueous solution was filtered using what man Filter Paper No. 1. The extract was stored at 4°C for future use. (Lee et al., 2014; Jha et al., 2016)

4.3. PRELIMINARY PHYTOCHEMICAL ANALYSIS

This preliminary phytochemical study detected the presence of steroids, alkaloids and glycosides in the aqueous extracts of *Smilax china* L. The plant constituents reportedly possessing hypoglycemic activity have been identified as alkaloids, flavonoids and related compounds, glycosides, steroids, terpenoids, polysaccharides, proteins and other miscellaneous compounds (Koti et al, 2009; Lamba et al, 2000).

TEST FOR REDUCING SUGAR

Benedict's Test Procedure

Begin by transferring 0.5 ml of an aqueous plant material extract into a test tube. Next, introduce 5 ml of Benedict's solution into the same test tube. Heat the mixture, allowing it to boil for a duration of 5 minutes, and then permit it to cool naturally.

Standard Fehling's Test Protocol

Commence the test by combining 2 ml of an aqueous plant material extract with 1 ml of a mixture created by equal volumes of Fehling’s solution A and B. Proceed to heat the resulting mixture, bringing it to a boil for a short period.

TEST FOR CARBOHYDRATES & GUMS

- **Molish test**

5 ml sample was taken and then a few drops of Molish reagent were added. Then the tube was inclined and 1 ml of sulphuric acid was added gradually at the bottom of the test tube through one side.

TEST FOR ALKALOIDS

- **Mayer’s test**

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a 1 ml of Mayer’s reagent was added.

- **Dragendroff’s test**

2 ml sample and 0.2 ml dilute hydrochloric acid were taken in a test tube. Then1ml Of dragendroff’s reagent was added.

- **Wagner’s test**

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of iodine solution was added.

- **Hager's test**

2 ml sample and 0.2ml of dilute hydrochloric acid were taken in a test tube. Then1 ml of picric acid was added.

TEST FOR TANNINS

- **Ferric chloride test**

5ml of solution of the extract was taken in a test tube. Then 1ml of 5% ferric Chloride solution was added.

- **Potassium dichromate test**

5ml of solution of the extract was taken in a test tube. Then 1ml of 10%
Potassium dichromate solution was added.

TEST FOR STEROIDS

- **Salkowski reaction**

A few mg of sample was dissolved in chloroform and a few drops of concentrated Sulphuric acid are added to the solution.

- **Liebermann - burchared reaction**

A few mg of sample was dissolved in chloroform and a few drops of concentrated Sulphuric acid are added to the solution followed by the addition of 2-3 drops of acetic anhydride.

TEST FOR FLAVONOIDS

A few drops of concentrated hydrochloric acid were added to a small amount of an alcoholic extract of the plant material.

TEST FOR SAPONINS

1ml solution of the extract was diluted with distilled water to 20ml and shaken in a graduated cylinder for 15 minutes.

4.4. ANTIMICROBIAL ACTIVITY:

Test Organisms

The test organism's were obtained and research work was done at Virtis Bio Labs Pvt. Ltd,39/3A2,Easwari Garden, Kesavan Nagar, kannankurichi, Salem,Tamilnadu-636 008.

AGAR WELL DIFFUSION METHOD

In the agar well diffusion technique, we began by inoculating Mueller-Hinton agar (MHA) plates with overnight cultures of both S. aureus and P. aeruginosa using the swabbing method. Following this, we created wells in the agar using a cork borer. Subsequently, we added 100 µL of various sample concentrations (ranging from 25 µg/mL to 400 µg/mL) into these wells. The plates were then left to incubate overnight at a temperature of 37°C. We carefully examined the zones of inhibition and measured their diameters. These results were subsequently compared to those obtained using the standard antibiotics, chloramphenicol (with a concentration of 30 mcg/disc) and ciprofloxacin (with a concentration of 5 mcg/disc).

MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

S.aureus and *P.aeruginosa* overnight growth bacterial culture was used. The extract was serially two-fold diluted in different concentrations in the range of (50 to 1600 µg/mL) for *S.aureus* and (50 to 800 µg/mL) for *P.aeruginosa* and also control without sample was incubated at 37°C for 24 hrs. The lowest concentration of extract that shows the absence of microbial growth is the minimum inhibitory concentration. From MIC well with no turbidity, 50 µL of aliquots was seeded in MHA agar plates and incubated at 37°C for 24 hrs. The lowest concentration which inhibits 100 % of bacterial growth was determined as the minimum bactericidal concentration.

4.5. BIOFILM ASSAY

The biofilm assay procedure commenced with the standardization of overnight cultures for both S. aureus and P. aeruginosa, adjusting them to an optical density of 0.4 at 600nm (OD600). These cultures were subsequently exposed to varying concentrations of the extract, ranging from 400 to 1600 µg/mL for S. aureus and 400 to 800 µg/mL for P. aeruginosa. Microtiter plates containing these cultures were then incubated for 24 hours at 37°C, with untreated cultures used as the positive control.

Following the incubation period, each well underwent a careful washing step with phosphate-buffered saline (PBS), after which they were fixed using 200 µL of 99% methanol for a duration of 15 minutes. After the removal of methanol, the bacterial biofilms were stained using a 0.1% crystal violet solution for a period of 15 minutes. Excess crystal violet was subsequently eliminated by rinsing with distilled water, and the plates were left to air-dry. To quantify biofilm formation, 99% ethanol was introduced to dilute the crystal violet adhered to

the biofilms. Ultimately, the optical density of the resulting solution was gauged at 570 nm using a microplate reader to assess biofilm formation.

4.6. EVALUATION OF CYTOTOXICITY

To evaluate cytotoxicity, we employed a modified MTT assay procedure, following the methodology established by Mossman in 1983. Initially, RAW 264.7 cells were seeded into 96-well plates at a density of 5×104 cells per well and incubated for a 24-hour period. After this initial incubation, the cells were exposed to various concentrations (ranging from 0.1 to 100 µg/ml) of an aqueous root extract extracted from Smilax china for an additional 24 hours.

For the MTT assay, we prepared a 5 mg/mL MTT solution in PBS and added 15 µL of this solution to each well. The cells were then incubated at 37ºC for 4 hours in an environment protected from light. Subsequent to this incubation, we meticulously aspirated the supernatant and solubilized the resultant formazan product in 200 µL of dimethyl sulfoxide (DMSO). Finally, we examined the plates' absorbance at 490 nm and 630 nm utilizing a multi-well microplate reader. Subsequently, we determined the inhibition rate employing the following equation:

$$\% \text{ Inhibition} = \{1- [A_{490} - A_{630} \text{ (Treated)} / A_{490} – A_{630} \text{ (control)}] \text{ x } 100\}$$

A - Absorbance

The value, i.e., the concentration required to inhibit 50% of cell viability was determined by plotting the log of the drug concentration versus the percentage of inhibition. The best-fit line was plotted by least square linear regression. The 50% inhibitory concentration (IC_{50}) was calculated from the linear-regression equation: Log (CV_{50}) = m×log(IC_{50}) +c; where m is the regression coefficient, c is the intercept of the line, log(IC_{50}) is the log of the 50%inhibitory concentration of the extract and log(CV_{50}) is the log value of 50% cell viability.

5. RESULTS

5.1. Preliminary phytochemical test

This preliminary phytochemical study detected the presence of steroids, alkaloids and glycosides in the aqueous extracts of *Smilax china* L. The plant constituents reportedly possessing hypoglycemic activity have been identified as alkaloids, flavonoids and related compounds, glycosides, steroids, terpenoids, polysaccharides, proteins and other miscellaneous compounds (Koti et al, 2009; Lamba et al, 2000).

Table 2: photochemical test for *smilax china* root extract.

GROUP TEST	NAME OF THE TEST	OBSERVATION
Carbohydrate & gums	Molish test	+
Reducing sugar	Fehling's test Benedicts test	+ +
Alkaloids	Mayer's Test Dragendroff's Test Wagner's Test Hagner's Test	- + - +
Steroids	Salkowski reaction Libermann-burchared reaction	+ +
Tannins	Ferric chloride test Potassium dichromate test	- -
Flavonoids	Hydrochloric acid test	-
Saponins	Foam test	-

+ Presence; - absence

5.2. EVALUATION OF ANTIMICROBIAL ACTIVITY BY AGAR WELL DIFFUSION METHOD

The antimicrobial properties of S. china root extract were evaluated using the agar well diffusion technique against S. aureus and P. aeruginosa. Figures 1 and 2, in addition to tables 3 and 4, present a comprehensive overview of the extract's antibacterial performance. The findings unequivocally reveal that S. china root extract displays significant efficacy in combating these gram-negative bacteria. The size of the inhibition zone directly correlates with microbial susceptibility, with susceptible strains exhibiting larger zones of inhibition, while resistant strains show smaller ones.

In order to ascertain the Minimum Inhibitory Concentration (MIC) of S. china root extract, a range of concentrations spanning from 25 to 400 µg/mL were examined. The MIC was identified at 100 µg/mL, where it achieved a 50% reduction in S. aureus growth compared to the control. Remarkably, at the highest concentration tested (400 µg/mL), the extract demonstrated complete inhibition, resulting in an 8 mm zone diameter, which is comparable to the performance of the standard drug Chloramphenicol. Consequently, the Minimum Bactericidal Concentration (MBC) was established at 400 µg/mL.

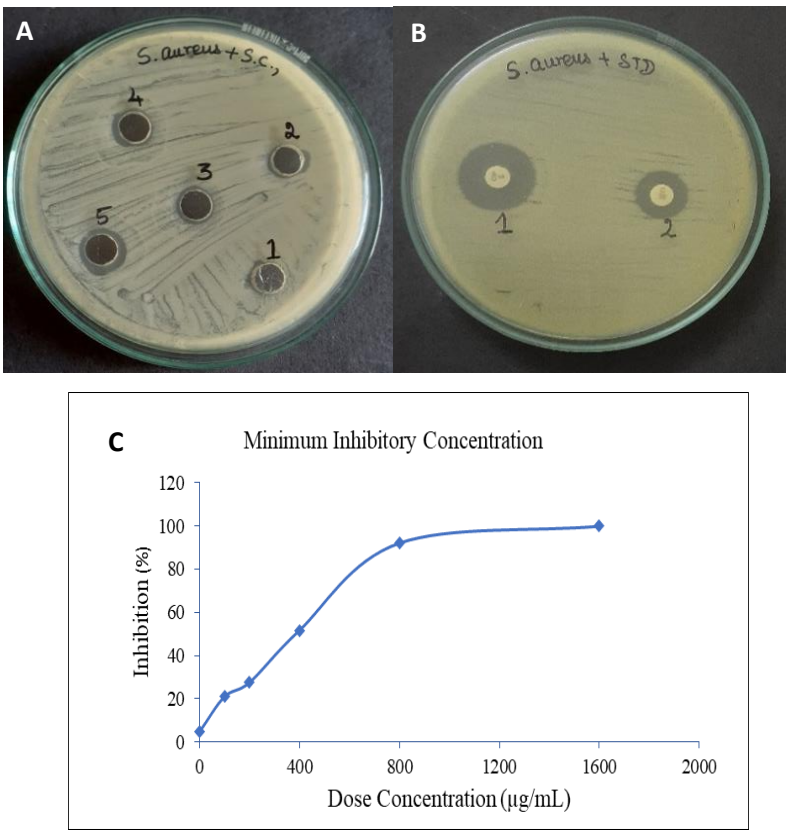


Figure 1: *S. aureus.*

A) Represents the zone of inhibition at different concentrations (25, 50, 100, 200, 400 µg/ml) of *S. china* root extract. B) Represents the zone of inhibition at different standard discs Ciprofloxacin (5 mcg) and Chloramphenicol (30 mcg). C) MIC of *S. china* root extract.

Table 3: Zone of inhibition of *S. aureus.*

Figure	Well No	*Smilax china* aqueous root extract concentration (µg/ml)	Zone of inhibition (in mm)
A	1	25	4
	2	50	6
	3	100	6
	4	200	7
	5	400	8
B	1	Ciprofloxacin (5 mcg/ disc)	7
	2	Chloramphenicol (30 mcg/disc)	10

The graph showed, the minimum inhibitory concentration of *Smilax China* ranging from 50 to 1600 µg/mL. The MIC was fixed as 800µg/mL which showed which showed more than 50 % inhibition of *S. aureus* compared to the control and the 1600 µg/mL showed 100% inhibition. 1600 µg/mL was fixed as MBC.

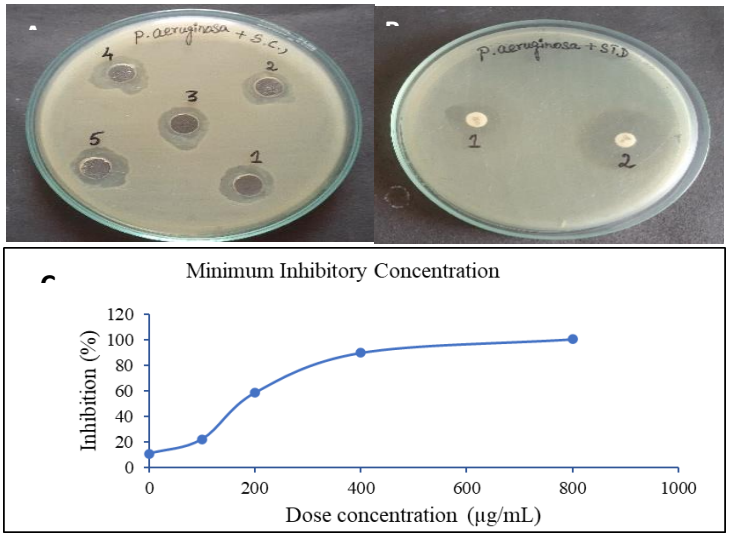


Figure 2: *P. aeruginosa.*

A) Represents the zone of inhibition at different concentrations (25 to 400µg/ml) of *S. china* root extract. B) Represents the zone of inhibition at different standard discs Ciprofloxacin (5 mcg) and Chloramphenicol (30 mcg). C) MIC of *S. china* root extract.

The graph showed, the minimum inhibitory concentration of *Smilax China* ranging from 50 to 800µg/mL. The MIC was fixed as 400µg/mL which showed more than 50 % inhibition of *P. aeruginosa* compared to the control and the 800 µg/mL showed 100% inhibition. 800µg/mL was fixed as MBC.

Table 4: Zone of inhibition of *P. aeruginosa.*

Figure	Well No	*Smilax china* root aqueous extract concentration (µg/ml)	Zone of inhibition (in mm)
A	1	25	6
	2	50	7
	3	100	8
	4	200	9
	5	400	10
B	1	Chloramphenicol (30 mcg/disc)	5
	2	Ciprofloxacin (5 mcg/ disc)	10

Graph in figure 2 showed that the minimum inhibitory concentration of *S. china* root extract ranges from 25 to 400 µg/mL. The MIC was fixed as 100µg/mL which showed 50% inhibition of *P. aeruginosa* compared to control and 400µg/mL showed 100% inhibition which indicates MBC.

5.3. BIOFILM ASSAY

Many bacteria can exist as surface-attached aggregations known as Biofilm. The micro titer plate biofilm assay is a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. Because it utilizes a 96-well plate format, it is suitable as a tool for screening large numbers of bacterial strains or species. *S. aureus and P. aeruginosa* biofilm formed on the flat-bottom wells after culture with or without *S. china* root extract (Figure 3) are quantified and evaluated. As the concentration of extract increased, the density of biofilm in both gram-negative bacteria decreased ($p \leq 0.01$, Figure 3 and 4). *S. aureus* biofilm yielded a dose response curve that quantifies the adhered biofilm biomass as a function of *S. china* root extract (Figure 3). This dose response curves can provide insights into the relative biofilm inhibition potency of the extract and can also indicate that the biofilm inhibition occurs at higher concentrations (1600 µg/ml).

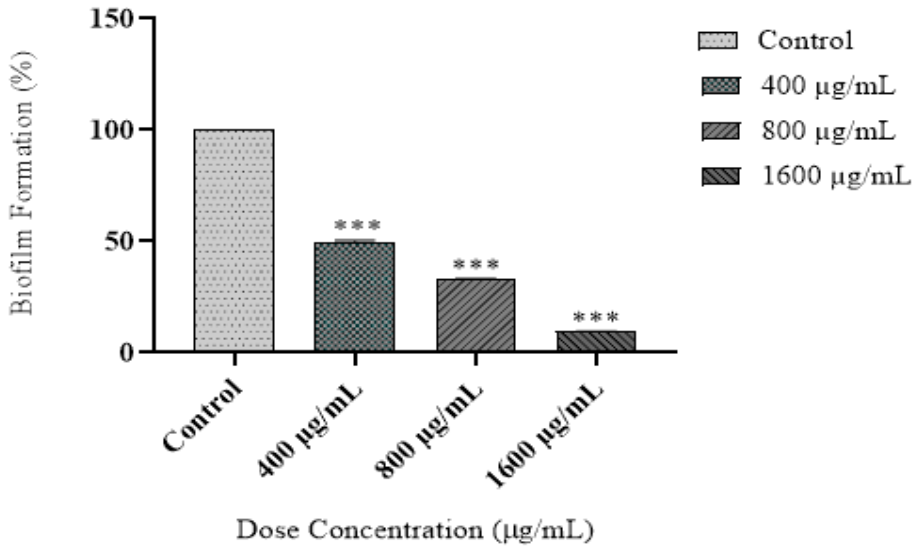


Figure 3*: S. aureus.*

Biofilm formed on the micro plate was less dense at a concentration of 800µg/mL root extract (Figure 4) in P. *aeruginosa*. This dose response curves can provide insights into the relative biofilm inhibition potency of the extract.

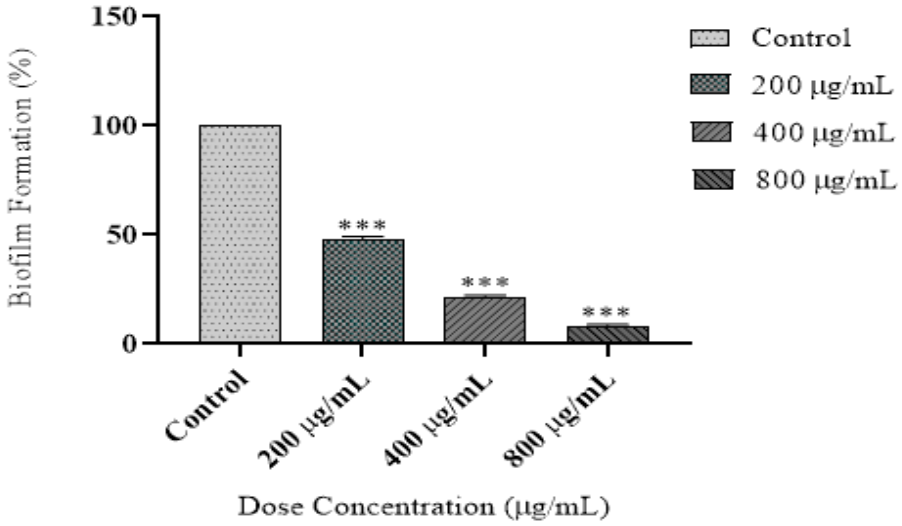


Figure 4: *P. aeruginosa.*

5.4. EVALUATION OF *IN VITRO* CYTOTOXICITY POTENTIAL BY MTT ASSAY

A cytotoxicity assay is employed to assess the potential for causing cellular or intercellular damage, including lethal cytotoxic effects. These assays are utilized by some researchers to predict potential harm to cells and tissues. To evaluate the impact of S.china root extract on RAW264.7 mouse macrophage cells, an MTT assay was conducted (see Fig. 5). The cells were exposed to various concentrations of S. china extracts ranging from 0.1 to 100µg/ml for a 24-hour period. The results indicated that S. china root extracts dose-dependently inhibited cell proliferation, implying that even at very low concentrations, the plant extract hinders the growth of RAW264.7 cells. The IC50 was calculated to be 3.1µg/ml, resulting in a 50.6% inhibition rate.

Table 5: Percentage inhibition and IC_{50} values of *S.china* against RAW 264.7 cells.

Sample	Concentration (µg/ml)	% Inhibition	IC_{50}(µg/ml)
Aqueous extract of *S.china*	0.1	6.020525	3.1
	0.2	12.67959	
	0.4	20.06842	
	0.8	34.04789	
	1.6	42.07526	
	3.125	50.66591	
	6.25	60.34208	
	12.5	76.89852	
	25	88.187	
	50	99.0878	
	100	99.63512	

6. DISCUSSION

In this investigation, we explored the potential antimicrobial properties of extracts derived from the roots of Smilax china L. against the bacteria S. aureus and P. aeruginosa. Our aim was to assess the suitability of these root extracts for potential pharmaceutical applications. While numerous studies have documented the antibacterial effects of Smilax china L. leaves and root extracts, there exists a scarcity of information regarding the utilization of Smilax china L. root extracts in biological contexts.

The escalating issue of antibiotic resistance has become a global concern, prompting extensive research efforts to combat this challenge (World Health Organization, 2014; Choski, A et al., 2019). This includes investigations into the use of antibiotics in animal production (Muaz, K et al., 2018). The Smilacaceae family comprises climbing shrubs, primarily represented by the genus Smilax, encompassing nearly 250 species worldwide, with approximately 26 species found in Mesoamerica (Huft, 1994). With a history of extensive usage since ancient times, prominent species within this genus include Smilax aristolochiaefolia Mill., S. febrifuga Kunth, S. ornata Hook, and S. regelii Killip & Morton, known for their roots and small rhizomes employed as antiseptic and anti-pruritic agents (British Herbal Pharmacopoeia, 1983). Smilax domingens Wild, native to Tropical America and commonly found in lowland areas within humid forests of broad-leaved species (Standley & Steyermark, 1952), is among the Smilax family. Notably, recent research has revealed the efficacy of Smilax china L. root extract in inhibiting bacterial growth. The medicinal plant Smilax china has yielded a plethora of secondary metabolites, including stilbenes and flavonoids such as oxyresveratrol, resveratrol, and scirpusin A (Kim, J.H et al., 2016; Shao et al., 2007), steroidal saponins (Shashida Y et al., 1992; Shao B et al., 2007; Tian et al., 2017), and cytotoxic phenylpropanoid glycosides (Feng et al., 2020; Huang, H et al., 2008; Jiang et al., 2021; Jiang, S.Y et al., 2019; Kong L et al., 2022; Lee, H.E et al., 2017; Shu et al., 2004). Moreover, novel phenylpropanoid glycosides, known as smilasides, have been isolated from the stems of Smilax china (Kuo Y.H et al., 2005).

Initial studies on the pharmacology of this particular species have revealed its potential as an antimicrobial agent. We have successfully verified its effectiveness against bacteria, aligning with one of its primary traditional uses for managing infections. Preliminary experiments have shown that crude extracts from the roots of Smilax china L. possess antimicrobial properties, particularly against Gram-negative bacteria. The compounds present in Smilax

china L. extracts are known for their varying degrees of antimicrobial activity. It's worth noting that the combined action of phenolic compounds, as observed in many cases, can significantly bolster their antimicrobial effects. A review of existing literature has emphasized the need for further research into the antibacterial properties of this plant.

In assessing the antibacterial capacity of Smilax china L. extracts, we measured the zone of inhibition and compared it to established antibiotics like ciprofloxacin and chloramphenicol. Even at a concentration of 200 µg/mL, we observed a zone of inhibition measuring 7 mm, which was in close proximity to the 5 mcg/disc zone produced by ciprofloxacin against S. aureus. This demonstrates a notable antibiotic potential at a moderate dosage when compared to conventional antibiotics. Similarly, at a concentration of 25 µg/mL, we observed a zone of inhibition of approximately 6 mm, surpassing ciprofloxacin and matching chloramphenicol's 30 mcg/disc zone against P. aeruginosa. These findings indicate that the zone of inhibition increases with a higher concentration of the plant extract. The presence of such inhibitory zones unequivocally suggests the plant's ability to disrupt bacterial membranes and act as an antibiotic. Furthermore, the minimum inhibitory concentration (MIC) of 400 µg/ml exhibited by these root extracts signifies significant antibacterial activity. These results underscore the plant extracts' effectiveness in combating bacteria, owing to a high concentration of bioactive components within them.

Phytoconstituents found in Smilax china L. root extract, including flavonoids, phenylpropanoid glycosides, and various biomolecules, are thought to contribute to their strong adsorptive binding to bacterial cell surfaces. This attachment of phytoconstituents may enhance the extract's antibacterial properties by potentially rupturing bacterial membranes, leading to the release of internal components and eventual bacterial cell death. Recent research by Ji-Hae Joo and colleagues in 2022 demonstrated the extract's potent antimicrobial activity against two strains of Cutibacterium acnes, with a more pronounced effect on gram-negative bacteria. This could be due to the thinner cell walls of gram-negative bacteria, allowing easier penetration of the extract into their cell membranes, causing damage (as suggested by Shehabeldine et al. in 2022).

Moreover, the aqueous extract of S. china exhibited notable cytotoxicity against RAW 264.7 cells. Several bioactive compounds responsible for this activity have been identified in the literature. For instance, Xie et al. in 2018 isolated seven new furostanol saponins (1-7) and nine known furostanol saponins (8-16) from Smilax china L. They found that compounds 1,

4, 6, and 11 significantly inhibited TNF-α mRNA expression in LPS-induced RAW264.7 cells, indicating potential anti-inflammatory properties.

Additionally, it's worth noting that crude Smilax china L. root extract displayed strong anti-biofilm activity. Microbial biofilms, composed of sticky exopolymeric substances, are responsible for microbial adhesion and antimicrobial resistance due to factors like eDNA, exoenzymes, limited antimicrobial diffusion, and nutrient scarcity. Surface proteins and polysaccharide intercellular adhesions play a role in biofilm formation. Detecting and combatting biofilms is crucial in addressing chronic infections, especially as they contribute significantly to hospital-acquired infections. Bacteria within biofilms are highly resistant to antibiotics, requiring substantially higher doses for eradication. Research into compounds, such as those found in S. china root extract, that can target biofilm-embedded bacteria has gained momentum. The extract's impact on biofilm detection methods was evaluated against Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus strains. Results indicated that the extract effectively reduced biofilm density in both strains in a dose-dependent manner, with inhibitory effects observed at concentrations higher than those required to inhibit bacterial growth. Future studies will be essential for a deeper understanding of biofilm formation mechanisms in various bacterial species, potentially identifying novel drug targets for future antibiofilm therapeutics.

7. CONCLUSION

In this research endeavor, our primary focus revolved around exploring the potential antimicrobial attributes inherent in root extracts derived from Smilax china L. Our aim was to assess their suitability for potential utilization in pharmaceutical applications, specifically in combating Staphylococcus aureus and Pseudomonas aeruginosa. The outcomes of our investigation unveiled notable antibacterial efficacy, underscoring the presence of a substantial concentration of bioactive constituents within these plant extracts. Furthermore, we observed that the aqueous extract from Smilax china exhibited significant cytotoxic activity when tested on RAW 264.7 cells. Interestingly, our findings also revealed that the inhibitory impact of Smilax china root extract on the biofilms of Pseudomonas aeruginosa and Staphylococcus aureus occurred at concentrations surpassing those necessary to inhibit bacterial growth. To advance our understanding, we advocate for future research endeavors dedicated to pinpointing the specific bioactive compounds responsible for each of these activities and elucidating the underlying mechanisms.

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