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INTEGRATED ANTIMICROBIAL, ANTIOXIDANT, PHYTOCHEMICAL, AND MOLECULAR DOCKING EVALUATION OF *ANDROGRAPHIS PANICULATA* EXTRACTS AGAINST MULTIDRUG-RESISTANT CLINICAL PATHOGENS

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ABSTRACT

This paper compared the antimicrobial, antioxidant, and phytochemical activity of *Andrographis paniculata* extracts with bacterial pathogens in the clinical samples. A total of twenty clinical samples (pus, urine, and sputum) produced different bacterial isolates such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Salmonella typhi*. Preliminary identification was made possible by Gram staining, cultural characteristics and biochemical tests, but the cart wheel assay confirmed multidrug resistance by using ethidium bromide efflux. *A. paniculata* aqueous and ethanolic extracts were obtained by means of maceration, and the phytochemical yield of the ethanol extract was higher. The evaluation of antibacterial activity using agar well diffusion showed that the ethanolic extract had broader inhibition zones, especially with *S. aureus* and *E. coli,* and synergistic mixtures gave stronger growth inhibition. MIC values also demonstrated that the ethanolic extract (3.125 -12.5 mg/mL) was more potent than the aqueous extract. The phytochemical screening established the presence of tannin, flavonoids, saponins, terpenoids, and steroidal constituents. The radical-scavenging ability of the ethanol extract was found to be more effective in the case of ethanol, which is attributed to the higher levels of phenolic compounds. TLC profiling has shown the presence of several metabolites, and GCMS analysis has detected fourteen key compounds, with the major constituents being piperine (38.67). CB-Dock molecular docking demonstrated positive binding affinities of the key phytochemicals and multidrug-resistant bacterial target proteins. All in all, the research confirms A. paniculata as a potential source of antimicrobial and antioxidant properties, and the ethanolic extract has high efficacy with a good potential for development as a therapeutic agent.

KEYWORDS: MDRB- Multi-Drug Resistant Bacteria, *Andrographis Paniculata*, Anti-synergistic activity, MIC, ABTS, DPPH assay, Molecular docking.

1. INTRODUCTION

The era of antibiotics started in 1928 when penicillin was discovered, and it significantly changed the healthcare system of the world and caused fewer deaths related to infectious diseases. Nonetheless, years of improper, overuse and uncontrolled use of antibiotics have hastened the development of multidrug-resistant bacteria (MDRB), so the previously curable diseases are now becoming harder to control [1]. MDR pathogens have resistance against different sets of antibiotics by mechanisms that include transfer of resistance genes on mobile plasmids or mechanisms of broad-spectrum efflux pumps that secrete various antimicrobial agents, a process referred to as cross-resistance. Another very worrying fact is the increasing rate of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, which inactivate major β-lactam-based antibiotics [2]. The ESBL-related infections are challenging to treat, and they are rapidly spreading; in certain areas of Southeast Asia, the percentage of the community of an estimated 50 people may be an asymptomatic carrier of the bacteria; this presents a significant future risk of resistant infections and the extensive spreading of the resistance genes.

Due to this epidemic, medicinal plants have been of increased interest as a source of new antimicrobial compounds. Formulations that are derived from plants gathered an immense attention than commercial antibiotics; for example, neem-based, nanoemulsion has showed increasing bacterial activity against pathogenic bacteria: *Vibrio anguillarum* [3].

Acanthaceae *Andrographis paniculata* has extensive Ayurvedic, Siddha, Unani, Homoeopathic, and Traditional Chinese medicine history because of its varied medicinal uses. The plant has antipyretic properties [4], cholagogue properties, hepatoprotective properties [5], and anti-inflammatory properties [6]. It finds wide application in the treatment of intermittent fevers such as dengue, malaria, chikungunya and chronic febrile diseases.

Recent reports indicate that its bioactive components, especially andrographolide, have a potent antimicrobial, immunomodulatory and antioxidant effect, which makes it a promising drug in the management of MDR bacterial infections. Nevertheless, its therapeutic importance should be weighed against serious consequences, where overconsumption can result in gastrointestinal discomfort, anorexia, emesis and possible reproductive impacts. It should not be used in pregnant and lactating women, and in those with hypotension, hyperacidity, duodenal ulcers, and bleeding disorders.

Molecular docking has become one of the potent computational techniques in recent years to supplement experimental antibacterial screening. The interaction patterns and binding affinity of phytochemicals and bacterial target proteins are predictable through docking, which can explain the possible action modes of antimicrobial activity. Molecular docking is important in confirming experimentally observed activity as well as the identification of possible lead compounds by identifying favourable ligand-protein interactions. CB-Dock is used to enable cavity-guided, blind docking to quickly determine a way in which bioactive metabolites of A. paniculata can target major key enzymes or structural proteins involved in bacterial pathogenicity and drug resistance [7],[8]. Given the urgent need for safe, plant-based alternatives to combat antibiotic resistance, scientific evaluation of *A. paniculata* is essential. Therefore, this study investigates the antibacterial, anti-synergistic, and antioxidant activities of aqueous and ethanolic extracts of *A. paniculata* against clinically isolated MDR bacteria and further characterises their phytochemical constituents through chromatographic, spectroscopic, and molecular docking analyses.

2. MATERIALS AND METHODS

2.1. Sample Processing, Culture Preparation, and Bacterial Isolation:

A total of 20 clinical sample specimens (pus, urine and sputum) were provided by Malar Clinical Laboratory, Kelambakkam and *Andrographis paniculata* powder used in this study was taken from Siddha Central Research Institute, Arumbakkam. All specimens were collected over the sampling time frame. Bacterial growth for the clinical samples was initially inoculated into nutrient broth, after which incubated overnight to allow for early bacterial multiplication [9]. The nutrient broth was consistently poured and sterilised by autoclaving at 121° C and 15 psi for 20 min for total decontamination. Pure nutrient agar medium was subsequently prepared according to normal instructions in distilled or deionised water heated until the solid particles were completely dissolved, and it was autoclaved under the same conditions for sterilisation. After cooling, the melted medium was poured into sterile Petri plates and cooled under aseptic conditions. After solidification, plates were labelled and inoculated by the streak plate method to have pure colonies.[10]. The inoculated plates were incubated at 37 °C, and resultant bacterial isolates were subcultured on nutrient slants for future use and further analysis [11]

2.2 Cart Wheel Method:

The cart wheel method was employed to assess whether the bacterial isolates exhibited multidrug resistance based on efflux pump activity. Overnight cultures grown in nutrient broth were adjusted to a 0.5 McFarland standard prior to testing. Trypticase Soy Agar (TSA) plates supplemented with 20 µL of ethidium bromide (EtBr) were freshly prepared on the day of the assay or the preceding day. Each plate was divided into twelve radial sectors to form the characteristic cart-wheel pattern as described [12]. The standardized bacterial suspension was swabbed along the pre-marked sectors following the procedure outlined [13]. The inoculated TSA–EtBr plates were incubated at 37 °C for 16 hours and subsequently examined using a gel documentation system or under UV illumination. Fluorescent emission from the colonies indicated active efflux of EtBr, confirming the presence of multidrug-resistant bacteria (MDRB), whereas the absence of fluorescence suggested non-MDR phenotypes [14].

2.3 Gram Staining Protocol and Morphological Characterization of Bacterial Isolates:

We used Gram staining, the origin in differential staining technique introduced by Christian Gram to differentiate bacterial groups based on differences in cell wall structure. The procedure was initiated by making a thin bacterial smear on a clean glass slide with an inoculating loop; the smear was then heat-fixed for proper attachment of cells. The slide was then flooded with the primary stain (crystal violet) for 60 seconds and then rinsed slightly with water. We then used mordant Gram's iodine, which was allowed to stand for one minute to fix the dye [15]. For the crucial decolourisation process, we only processed the slide by dipping it briefly in ethanol or acetone with a rinse to avoid over-decolourisation [16]. Colonial morphology of the purified bacterial isolates was observed and recorded on selective media for their useful characters, i. e., colony colour, texture and appearance, to help identify species after the staining.[17].

2.4 Aqueous and Ethanolic Extraction of *Andrographis paniculata*

Aqueous and ethanolic extracts of *Andrographis paniculata* were prepared using standard maceration techniques. For the aqueous extract, 100 g of dried plant powder was mixed with 1000 ml of distilled or deionised water in a conical flask and agitated on a rotary shaker at 120 to 140 rpm for 72 hours. Whatman filter paper was then used to filter the mixture and to obtain the filtrate, which was then evaporated on a water bath at 95°C to obtain a concentrated extract that was further used [18]. In the case of the ethanolic extract, 100 g of plant powder was macerated in 1000 ml of absolute ethanol

and stirred and shaken under the same conditions of speed throughout the entire 48 hours, and the extraction cycle was repeated until the plant material was exhausted. The pooled filtrates were split into two; one was concentrated at 50º C with a Laborota 4000 rotary evaporator to extract phytochemicals but the other portion was evaporated in a water bath at 95 °C to obtain a thick extract. All the dried ethanol extracts were stored at 4 o C to be subjected to further experimental procedures [19].

2.5 Antibacterial and Synergistic Activity Testing

The antibacterial properties of the plant extracts and their fractions were assessed using the agar well diffusion technique. Before testing, bacterial inoculum was adjusted to 0.5 McFarland turbidity (0.60 x 10^{8} CFU/mL; 0.15 x 10^{8} CFU/mL). Mueller-Hinton agar (MHA) was made by dissolving the necessary quantity of medium in distilled water, after which it was sterilised by autoclaving. The sterile medium was then poured into Petri plates and left to solidify, and the standardised bacterial cultures put uniformly on the surface by use of the spread plate technique. Aseptically prepared wells were prepared in agar, and 100 µL of each plant extract or fraction (100 mg/mL stock) was added to the respective wells. Chloramphenicol and erythromycin (1mg/ mL) were used as positive controls, whereas DMSO was used as a negative control to make sure that the solvent was free of any intrinsic antibacterial activity. After 24 hours of incubation, the diameters of the inhibition zones were recorded and compared to the reference antibiotics to determine the relative antimicrobial activity [20]. The interactive synergies of the plant extracts were also studied through combining extracts mix in a 1:1 proportion and testing them with the same well diffusion test. The measure of synergy was the growth inhibitory index that was determined as a ratio between the inhibition zone of the combination and the added inhibition zones of the agents [21].

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of crude extracts and fractions was determined by a micro dilution procedure. Bacterial inoculum was raised in nutrient broth, and stock solutions of the plant extracts (50 mg/mL) in DMSO were obtained. Microtiter serial dilutions were done to yield concentrations of 50 mg/mL through to 0.390 mg/mL. A total of 100 µL of standard bacterial inoculum was added to every well. They were control wells: broth + extract (extract control), broth + bacteria (bacterial control), and broth only (negative control). A 24-hour incubation with plates being kept at 37 °C was then noted and the lowest concentration of extract that prevented visible bacterial growth was recorded as the MIC [22].

2.7 Phytochemical Analysis of Plant Extracts

2.7.1 Detection of Tannins, Phlobatannins, and Saponins: The crude extract was subjected to the presence of tannins by boiling 0.5 g of the dried extract in 20 ml of water, settling the mixture and adding 0.1% ferric chloride to the filtrate. The appearance of brownish-green or blue-black colouration was a positive result for tannins. Phlobatannins were analyzed by boiling 1 g of extract with 1% aqueous hydrochloric acid; the appearance of a red precipitate specific to phlobatannins permitted their identification. Saponins were tested by boiling 2 g of extract in 20 ml of distilled water, filtering, and vigorously shaking 10 ml of filtrate with 5 ml of water to watch continued frothing. Detection of Flavonoids and Steroids 2.7.2 Preparation of an emulsion with the addition of olive oil was an additional confirmation of saponins, as the appearance of a colored solution, i.e., yellow, was a positive outcome. Steroids were identified by dissolving 0.5 g of ethanolic extract in acetic anhydride, and then cautiously adding concentrated sulfuric acid; the development of a green, violet, or blue colour was used to determine the presence of steroidal compounds.

2.7.2 Detection of Terpenoids and Glycosides: Terpenoids were detected by the Salkowski test, which consisted of mixing 5 ml of crude extract with chloroform and layering concentrated sulfuric acid along the wall of the test tube. A reddish-brown colour at the interface confirmed the presence of terpenoids. The Keller-Kiliani test was conducted to identify glycosides, in which 5 ml of extract was combined with glacial acetic acid and ferric chloride, drop after concentrated sulfuric acid. A brown ring at the interface was a sign of the existence of cardiac glycosides.

2.8 ABTS Assay for Determination of Antioxidant Capacity

The ABTS assay was done in accordance with a standard spectrophotometric procedure to determine the antioxidant capacity. ABTS was dissolved in 100 mM sodium acetate buffer to prepare the working substrate solution. A cuvette was filled with 3 ml of this substrate and placed in the UV–visible spectrophotometer. An aliquot (20–100 µl) of enzyme extract from the fermentation broth was added directly into the cuvette, and the stopwatch was started immediately upon mixing. Absorbance readings at 420 nm were recorded at one-minute intervals to monitor the rate of oxidation [23].

Laccase Activity Formula:

$$\frac{(Absorbance\ at\ 420\ nm \times Volume\ of\ reaction\ mixture\ [ml] \times Dilution\ factor)}{(Molar\ extinction\ coefficient\ of\ ABTS\ at\ 420\ nm \times Volume\ of\ enzyme\ in\ reaction\ mixture\ [ml] \times Incubation\ time)}$$

2.9 DPPH Radical Scavenging Assay

2.9.1 Preparation of DPPH Solution and Sample Incubation: The DPPH working solution was prepared by weighing 10 mg of DPPH salt on aluminium foil and dissolving it in 20 mL of absolute ethanol in a conical flask. An additional 20 mL of ethanol was added to bring the total volume to 40 mL. The absorbance of this solution was measured at 517 nm using a UV spectrophotometer, and the value was adjusted to fall within the range of 1.08–1.12. If the absorbance exceeded 1.12, the solution was diluted with ethanol; if it was below 1.08, additional DPPH salt was incorporated to correct the concentration. An incubation period was introduced to stabilise the reaction. To prepare the sample, 1.5 mL microtubes

were covered with aluminium foil so that the tube was not exposed to light. The samples were set in pairs, at least one of them containing a control. Reactions were set up using the following volumes: for the control, 20 µL of absolute ethanol and 280 µL of DPPH solution; for the test samples, 10 µL of ethanol, 280 µL of DPPH, and 10 µL of sample extract. The mixtures were homogenised by gentle pipette mixing and incubated in darkness at 25 °C for 24 hours.

2.9.2 Calculation of DPPH Radical Scavenging Activity.

The percentage of DPPH radical inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{Abs_{control} - (Abs_{sample} - Abs_{blank})}{Abs_{control}} \times 100$$

Where.

- $Abs_{control}$ = Absorbance of DPPH solution
- Abs_{sample} = Absorbance of the sample with DPPH
- Abs_{blank} = Absorbance of ethanol (negative control).

2.10 Thin Layer Chromatography (TLC)

2.10.1 Analysis of Phytochemical Components. Thin Layer Chromatography (TLC) was employed to identify the biochemical and phytochemical constituents, such as alkaloids, phospholipids, and amino acids, in the plant extract. For the analysis, TLC plates were cut to approximately 8 cm in length, and two reference lines were drawn: one at the bottom (1 cm) and one near the top (30 mm) of the plate. Plant extract was applied to the baseline, and the plates were developed in a mobile phase consisting of petroleum ether and ethyl acetate. These spots were identified and later spotted by employing other detection reagents, such as immersion in a potassium permanganate solution, iodine gas in an iodine chamber, and UV spectrophotometry, which has facilitated the identification of components [24]. The factor (Rf) was calculated by dividing the distance traversed by the compound and the distance traversed by the front of the solvent, which offered quantitative evidence in recovering the organic compounds in the mixture.

$$R_f \text{ Value} = \frac{Distance\ travelled\ by\ compound}{Distance\ travelled\ by\ solvent\ front}$$

2.11 Identification of Bioactive Compounds Using GC-MS

The plant extracts were also found by Thin Layer Chromatography (TLC) to contain bioactive compounds and this was again verified by Gas Chromatography-Mass Spectrometry (GC-MS). In the case of GC-MS, we injected the samples into an HP-5 column (30 m x 0.25 mm ID, 0.25 mm film thickness) with an Agilent Technologies QP2010 Ultra GC-MS system (model 122-5532, 122-5631). Mass spectrometry conditions were as follows helium as a carrier gas with flow rate of 1 mL/min, ionizer temperature was 250° C, and mass range used was 50-600 m/z [25] Compound identification, the mass spectra of the samples were compared to standard spectra in the National Institute of Standards and Technology (NIST) database of over 62,000 patterns, as well as NIST 17 and Wiley 8 libraries.

2.12 Molecular Docking Analysis

Molecular docking was carried out using the CB-Dock server, which employs curvature-based cavity detection followed by AutoDock Vina–driven blind docking to predict optimal ligand–protein interactions [26]. Phytochemicals of *Andrographis paniculata* were retrieved from PubChem, converted to PDB format, and energy-minimised using MMFF94. Target multidrug-resistant bacterial proteins were downloaded from the Protein Data Bank and prepared by removing water molecules and heteroatoms, adding polar hydrogens, and optimising charges. Each ligand–protein pair was docked on CB-Dock, which automatically generated binding cavities and Vina scores, and the best pose was selected based on the lowest binding energy. PyMOL and Discovery Studio were used to visualise all the docked complexes to assess the presence of hydrogen bonds, hydrophobic interactions, and participating residues. It is an optimised workflow that corresponds to the methodologies of virtual screening and is supported by the docking reliability that has been validated in CB-Dock and AutoDock Vina benchmarking experiments [27].

3. RESULTS

3.1 Sample Processing, Culture Preparation, and Bacterial Isolation

Out of 20 clinical specimens (pus, urine, and sputum), the bacteria were observed to have grown in the presence of nutrient broth after incubated overnight. Streak plating resulted in colonies which were well separated, thus showing the separation between mixed cultures. Nutrient agar plate sub-culturing and slant sub-culturing led to stable pure colonies.

3.2 Cart Wheel Method

The cart wheel test was able to distinguish the isolates according to the activity of the efflux pump. Following the incubation period of 16 hours in the TSA plates that were supplemented with ethidium bromide, some bacterial isolates exhibited a high level of fluorescent emission under UV light. The presence of the multidrug-resistant (MDR) phenotypes in the strains tested was confirmed by the presence of the EtBr effluxing colonies. Conversely, a group of isolates exhibited minimal or no fluorescence and thus weak or no efflux pump activity and were hence termed as non-MDR strains. The strength of fluorescence across the MDR isolates was not the same, and this indicated that the efflux pump efficiency may vary across species or strains. In sum, the cart wheel technique was able to differentiate MDR isolates compared to non-resistant isolates using unambiguous and interpretable phenotypic profiles (Table 1).

Table 1: Interpretive Cart wheel results of screening for 20 tested isolates.

Isolate ID	Interpretation	Isolate ID	Interpretation
Bac 01	**Positive**	Bac 11	**Positive**
Bac 02	Negative	Bac 12	Negative
Bac 03	Negative	Bac 13	Negative
Bac 04	Negative	Bac 14	Negative
Bac 05	Negative	Bac 15	**Positive**
Bac 06	Negative	Bac 16	Negative
Bac 07	Negative	Bac 17	Negative
Bac 08	Negative	Bac 18	Negative
Bac 09	Negative	Bac 19	**Positive**
Bac 10	Negative	Bac 20	Negative

3.3 Gram Staining Protocol and Morphological Characterization of Bacterial Isolates.
Gram staining identified the isolates as Gram-positive cocci and Gram-negative bacilli, which was in line with typical clinical pathogens. *Staphylococcus* spp. were seen in the form of violet-stained cocci in clusters, *Bacillus subtilis* were seen in the form of violet-stained rods, whereas *E. coli, Klebsiella* and *Pseudomonas* were seen as pink colored rods. Characteristic features of colonies such as colour, elevation, margin, and surface texture and biochemical tests matched the morphological and biochemical profiles as provided by the standard criteria, which indicated the successful primary identification (Table 2).

Table 2. Morphological and biochemical characterization of bacterial isolates.

Isolate ID	Gram Reaction	Selective Medium & Colony Morphology	Typical Biochemical Characteristics	Presumptive Identification
Bac 01	Gram-negative rods	**MacConkey Agar:** Appearance of pink colonies (Lactose fermenting)	Indole (+), Methyl Red (+), Voges-Proskauer (-), Citrate (-)	*Escherichia coli*
Bac 07	Gram-positive rods	**Nutrient Agar:** Rough, opaque, fuzzy white colonies	Catalase (+), Citrate (+), Starch Hydrolysis (+)	*Bacillus subtilis*
Bac 11	Gram-negative rods	**SS Agar:** Smooth and opaque colonies	Motility (+), H_2S production (+), Urease (-), Indole (-)	*Salmonella typhi*
Bac 15	Gram-negative rods	**Cetrimide Agar:** Greenish colour pigmentation	Oxidase (+), Catalase (+), Citrate (+), Nitrate Reduction (+)	*Pseudomonas aeruginosa*
Bac 19	Gram-positive cocci	**EMB Agar:** Yellow colonies with yellow zones	Catalase (+), Coagulase (+), Mannitol Fermentation (+)	*Staphylococcus aureus*

3.4 Aqueous and Ethanolic Extraction of Andrographis paniculate.
Following filtration and solvent evaporation, both aqueous and ethanolic extractions gave dark green semi-thick crude extracts. A greater volume of the aqueous extract was obtained, whereas the ethanol extract had a lower viscosity, indicating the product was efficiently extracted with alcohol-soluble phytochemicals, giving a more concentrated and aromatic residue. The extracts were stored at 4^{o} C during the study.

3.5 Antibacterial and Synergistic Activity Testing
The agar well diffusion analysis revealed that both the aqueous and ethanol extracts had a quantifiable antibacterial effect, with the ethanol extract providing bigger zones of inhibition. The most susceptible isolates were *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa, which* showed relatively reduced sensitivity, indicating active synergistic interactions between extract components. Synergistic testing showed that extracts combined in a 1:1 ratio yielded increased inhibition zones with a positive Growth Inhibitory Index (>1) (Table 3).

Table 3. Antimicrobial activity of *Andrographis paniculata* extracts against selected bacterial pathogens.

Test Organisms	Zone of inhibition [mm]		
	Aqueous extract	**Ethanolic extract**	**DMSO (Control)**
Pseudomonas aeruginosa	7	10	–
Bacillus subtilis	–	–	–
Escherichia coli	6	8	–
Staphylococcus aureus	15	7	–
Salmonella typhi	–	–	–

3.6 Determination of Minimum Inhibitory Concentration (MIC).
MIC analysis revealed that ethanolic extracts had lower MIC values (high potency) than aqueous extracts of all isolates. The MIC values were found to be 3.125-12.5 mg/mL for the ethanol extracts and 12.5-50 mg/mL for the aqueous extracts. The maximum concentration to inhibit *Pseudomonas aeruginosa* was always the largest (Table 4).

Table 4. Minimum Inhibitory Concentration (MIC) of *Andrographis paniculata* extract against selected bacterial pathogens.

Test Organisms	MIC [mg/mL]
Pseudomonas aeruginosa	70
Bacillus subtilis	70
Escherichia coli	50
Staphylococcus aureus	50
Salmonella typhi	90

3.7 Phytochemical Analysis of Plant Extracts
3.7.1 Detection of Tannins, Phlobatannins, and Saponins: It was determined that the following compounds were present: Tannins: blue-black colour with ferric chloride. Phlobatannins: precipitates red in the presence of acid. Saponins: stable emulsion of olive oil and persistent froth. These findings confirmed the existence of several water-soluble bioactive compounds.

3.7.2 Detection of Flavonoids and Steroids: A clear yellowish colouration was a positive indication of flavonoids in the aqueous extract. The extracts using ethanol were green to blue in colour, proving the presence of steroidal compounds. These results indicate that there is strong antioxidant and antimicrobial phytochemical makeup.
3.7.3 Detection of Terpenoids and Glycosides: The interface of the Salkowski test was reddish-brown, indicating the presence of terpenoids, whereas the result of the Keller-Kiliani test was a brown ring, indicating cardiac glycosides. These substances enhance the therapeutic and medicinal effects of the extract (Table. 5).

Table 5. Phytochemical screening of *Andrographis paniculata* extracts

Phytochemical Constituent	Extract	Observation	Inference
Tannins	Aqueous	Blue-black coloration	+
Phlobatannins	Aqueous	No red precipitate	–
Saponins	Aqueous	Stable emulsion formed	+
Flavonoids	Aqueous	Yellow coloration	+
Steroids	Ethanolic	No violet-green coloration	–
Terpenoids	Aqueous	Reddish-brown coloration	+
Glycosides	Aqueous	No brown ring	–

3.8 ABTS Assay for Determination of Antioxidant Capacity
The ABTS assay was used to indicate that the extracts had potent antioxidant capacity, with the ethanolic extract having higher reduction in absorbance at 420 nm with time. The computations of Laccase activity revealed that the ethanolic extract was more effective in radical-scavenging because the antioxidant compounds were more soluble in alcohol (Figure

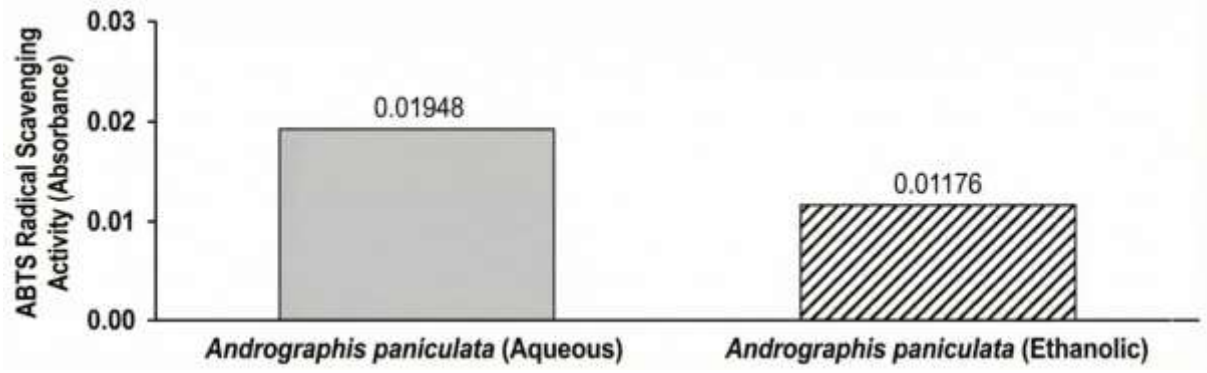

Figure 1. Comparison of ABTS radical scavenging activity between aqueous and ethanolic extracts of *Andrographis paniculata*.

3.9 DPPH Radical Scavenging Assay

3.9.1 Preparation of DPPH Solution and Sample Incubation. The DPPH working solution was stable in the absorbance range of 1.08-1.12, which was needed. All the samples that were incubated for 24 hrs in darkness showed observable colour reduction, which means that there were electron-donation reactions.

3.9.2 Calculation of DPPH Radical Scavenging Activity. Both extracts had dose-dependent scavenging activity, with ethanolic extract giving the highest percentage inhibition. The use of blank and control provided proper background correction. The most active ones were the samples containing greater amounts of phenolics (Figure 2).

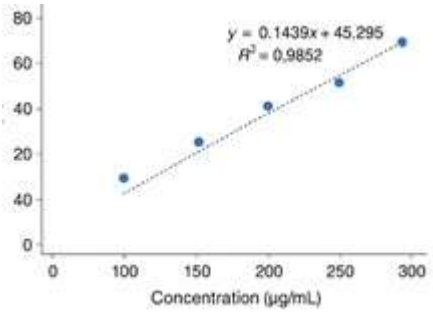

Figure 2. Linear correlation between sample concentration and RSA (%) in water ($R^2 = 0.9852$).

3.10 Analysis of Phytochemical Components using TLC

Under UV light and chemical visualisation, TLC showed clear, well-separated spots, which showed that there were multiple phytochemicals. The alkaloids, flavonoids, terpenoids, and other metabolites were identified by way of differently colored spots. The Rf values were 0.22 to 0.87, indicating the multiplicity of the compounds extracted in *A. paniculata*.

3.11 Identification of Bioactive Compounds Using GC-MS

The ethanolic extract was analysed by GC-MS, which identified 14 dominant phytochemical constituents after being identified by retention time and spectral similarity with NIST/Wiley libraries. In the chromatogram, several peaks were observed, which showed that there was a chemically enriched extract. The most common compound (R.T. 43.129; 38.67% peak area) was Piperile, which is the largest bioactive component of the extract. Diethyl phthalate was the largest fraction (28.48%), then medium-abundance compounds, i.e., isopropyl linoleate (5.40%), ethyl oleate (3.43%), and saturated fatty acid derivatives, i.e., n-hexadecanoic acid and ethyl hexadecanoate (combined -6.55%) (Table 6). Some low-abundance compounds (0.83-3%), such as ketone products, phthalates, siloxanes, and spirocyclic solids, were also identified. Though these molecules are lower in percentages, they are synergetic to antioxidant and antimicrobial activities. In general, the GC-MS profile shows that it was a phytochemically rich extract with major bioactive secondary metabolites which are dominated by piperine (Figure 3).

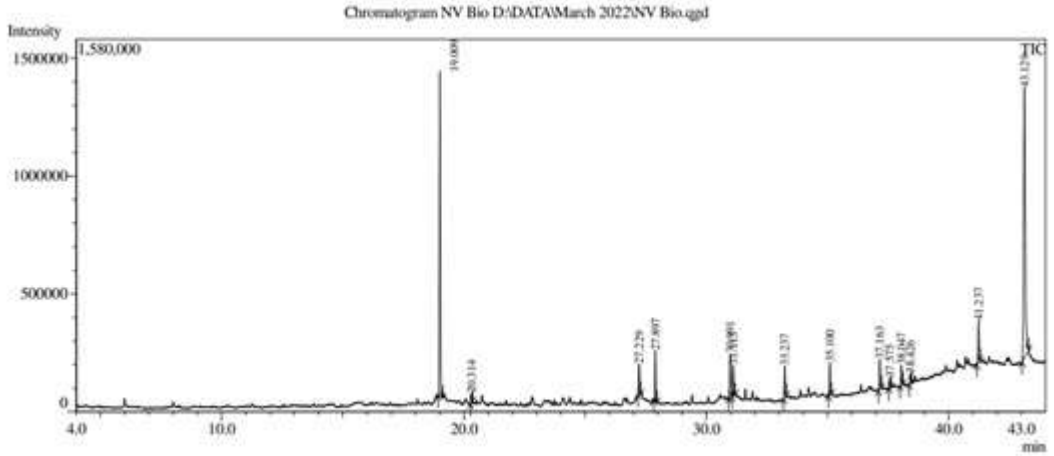

Figure 3. GC–MS total ion chromatogram (TIC) of the ethanolic extract of *Andrographis paniculata* (NV Bio). Major peaks corresponding to bioactive phytochemicals are detected across the retention window, with dominant peaks observed at 19.00 and 43.12 min.

Table 6. Chemical Profile: Identification of Piperine (38.67%) as the Major Constituent alongside Solvent Residues.

Peak #	R.Time	Area	Area %	Compound Name
1	19.009	3,813,331	28.48	Diethyl Phthalate
2	20.314	106,490	0.8	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-
3	27.229	350,706	2.62	n-Hexadecanoic acid
4	27.897	525,883	3.93	Hexadecanoic acid, ethyl ester
5	30.991	723,533	5.4	Isopropyl linoleate
6	31.113	459,720	3.43	Ethyl Oleate
7	33.237	396,283	2.96	1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one
8	35.1	372,866	2.78	Spiro[furan-2(5H),2'(1'H)-naphtho[2,1-b]furan]-5-one
9	37.163	379,072	2.83	Bis(2-ethylhexyl) phthalate
10	37.575	121,792	0.91	1,1,6-trimethyl-3-methylene-2-(...)-dimethyle
11	38.047	241,944	1.81	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-
12	38.426	131,314	0.98	Tetracosamethyl-cyclododecasiloxane
13	41.237	589,323	4.4	(1R,2R,8S,8Ar)-8-hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyl-cis-decalin
14	43.129	5,177,316	38.67	**Piperine**
Total		13,389,573	100	

3.12 Molecular Docking

Molecular docking of piperine to the NorA efflux pump of *Staphylococcus aureus and* MexB efflux pump of *Pseudomonas aeruginosa* has yielded five binding poses with scores varying between -8.3 to -4.9 kcal/mol and -8.1 and -7.3 kcal/mol (Tables 7 & 8). The highest scoring pose (–8.3 kcal/mol and -8.1 kcal/mol) was found deep into a hydrophobic binding pocket composed of key residues including Val175, Phe198, Met267, Leu297, Ile315 and Ser257 for NorA and Phe450, Pro40, Ala305, Val216, Gly273, Ile277, and Ser375 for MexB, in which it made stabilizing hydrophobic contacts, π–π interactions, and, in some cases, hydrogen bonds. Conversely, weaker surface-level interactions were demonstrated in lower-scoring poses. The high binding affinity to NorA indicates that piperine can easily interact with and possibly inhibit this efflux pump, which is a significant cause of multidrug resistance in *S. aureus* and a potential MexB efflux pump inhibitor that can diminish intrinsic multidrug resistance in *P. aeruginosa*. These results justify the use of piperine as a bioactive antimicrobial agent present in the GCMS pattern of *Andrographis paniculata* and its role in inhibiting efflux-mediated resistance, which should be further verified in vitro (Figure 4 & 5).

Table 7. Docking parameters and binding scores for piperine– NorA interactions

Vina score [kcal/mol]	Cavity size [Å³]	Center [Å]			Grid size [Å]		

		x	y	z	x	y	z
–8.3	543	–25	8	–12	23	23	23
–6.3	58	–29	12	–3	23	23	23
–5.8	26	–15	20	1	23	23	23
–5.0	31	–10	11	–3	23	23	23
–4.9	33	–11	26	–4	23	23	23

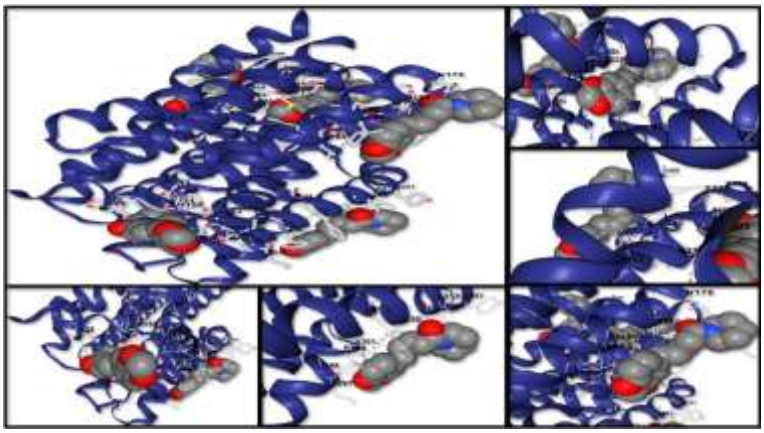

Figure 4. Molecular docking interaction of piperine with the NorA efflux pump of *Staphylococcus aureus.*

Table 8. Docking parameters and binding scores for piperine–MexB interactions.

Vina score [kcal/mol]	Cavity size [Å³]	Center [Å]			Grid size [Å]		
		x	Y	z	X	y	z
–8.1	11,986	167	208	218	35	35	24
–8.0	16,405	171	211	187	35	34	35
–7.8	9,691	184	200	238	35	24	32
–7.3	44,532	199	174	209	35	35	35
–7.3	4,029	196	165	193	32	32	24

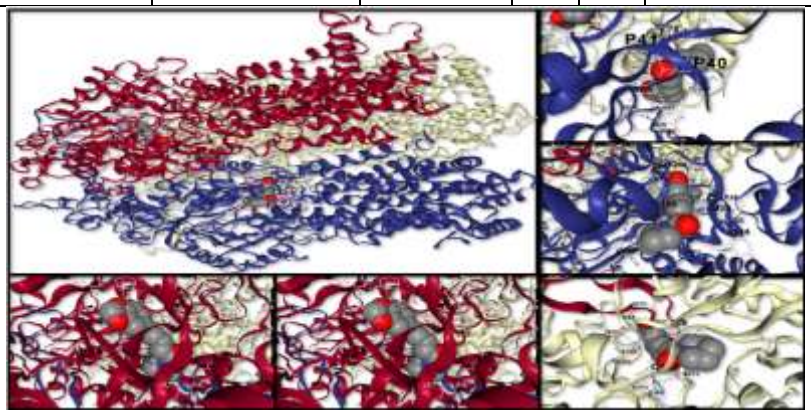

Figure 5. Molecular docking interaction of piperine with the MexB efflux pump of *Pseudomonas aeruginosa*

4. DISCUSSION

The successful isolation and screening of five bacterial pathogens from a clinical source was performed by standard microbiological protocol to retrieve different microbial loads. The occurrence of both Gram-positive and Gram-negative bacteria is consistent with recent publications highlighting that clinical infections are most often caused by *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*, which show multidrug resistance activity [28]

The cart wheel method using ethidium bromide acts as a good physical characteristic tool to detect efflux pump activity. Efflux complexes such as NorA and MexAB-OprM play an important role in drug resistance by actively eliminating drugs from bacterial cells [29]. The antibacterial activity of *Andrographis paniculata* extracts was shown in this study; specifically, the ethanolic extract's efficacy can be ascribed to enhanced extraction of bioactive phytochemicals like flavonoids, terpenoids, and alkaloids. Recent studies have shown that ethanol is more active than aqueous in extracting lipophilic antimicrobial compounds [30]. The synergistic antibacterial effects observed prove that the phytoconstituents act through numerous mechanisms, including membrane disruption and efflux pump inhibition. Plant-synthesised compounds have been widely reported as antibiotic adjuvants that can enhance antimicrobial efficacy [31]. MIC activity also assures the enhanced potential of ethanolic extracts. The greater resistance observed in Pseudomonas aeruginosa can be because of intrinsic resistance mechanisms such as low membrane permeability and efficient efflux systems [28]. Phytochemical activity revealed the occurrence of tannins, flavonoids, saponins, and terpenoids, which naturally have antimicrobial and antioxidant capacity. These compounds exhibit their activity through protein precipitation, membrane disruption and free radical scavenging [32]. TLC analysis also proved the presence of different phytochemicals in the extract. The antioxidant capacity calculated through ABTS and DPPH assays shows strong radical scavenging activity, specifically in ethanolic extracts. This is consistent with recent findings that phenolic compounds play a vital role in antioxidant defence systems [33]. GC-MS analysis disclosed a phytochemically rich extract, with Piperine detected as the major compound (38.67%). Piperine is well known for its antimicrobial, antioxidant, and bioenhancing properties, and recent studies have shown its capability to modulate bacterial resistance mechanisms [33]. Molecular docking studies demonstrated greater binding affinity of piperine with NorA and MexB efflux pumps, indicating its crucial role as an efflux pump inhibitor. The shown binding energies (-8.3 and -8.1 kcal/mol) indicate stable ligand-protein interactions. These innovations are related to the latest studies showing natural compounds can inhibit efflux pumps and restore antibiotic susceptibility [34],[35].

5. CONCLUSION

The present study can be concluded as the research generates a significant antimicrobial and antioxidant value of *Andrographis paniculata*, which is backed by analysis of the phytochemical, GC-MS, and molecular docking. Ethanolic extracts were more active as evidenced by larger inhibition zones, lower MIC, and higher ABTS /DPPH radical scavenging activity relative to aqueous extracts, which revealed cost-effective and economical extraction of phenolic and lipophilic bioactives. Analysis using GC -MS showed that its main and dominant constituent was piperine (38.67%), and at the same time, several fatty acid esters and terpenoid forms that played a role in bioactivity. Docking experiments also confirmed strong affinity of piperile to the NorA (*S. aureus*) and MexB (*P. aeruginosa*) efflux pumps (Vina scores: -8.3 and -8.1 kcal/mol), which are based on efflux-mediated resistance. All these results suggest the presence of *A. paniculata* and especially the presence of piperine-containing fractions as a potentially viable source in the design of an antimicrobial agent against multidrug-resistant strains.

8. REFERENCES

[1] Li X-Z, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs 2009;69:1555–623. https://doi.org/10.2165/11317030-000000000-00000.

[2] Vivas R, Barbosa AAT, Dolabela SS, Jain S. Multidrug-Resistant Bacteria and Alternative Methods to Control Them: An Overview. Microb Drug Resist 2019;25:890–908. https://doi.org/10.1089/mdr.2018.0319.

[3] Mani R, Thukkaram S, Sudhakaran R, Parameswaran V, Thyagarajan R. Antibacterial property of neem nanoemulsion against Vibrio anguillarium infection in Asian sea bass (Lates calcarifer) 2019.

[4] Aronoff DM, Neilson EG. Antipyretics: mechanisms of action and clinical use in fever suppression. Am J Med 2001;111:304–15. https://doi.org/10.1016/s0002-9343(01)00834-8.

[5] Rana AC, Avadhoot Y. Hepatoprotective effects of Andrographis paniculata against carbon tetrachloride-induced liver damage. Arch Pharm Res 1991;14:93–5. https://doi.org/10.1007/BF02857822.

[6] Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med 1998;104:2S-8S; discussion 21S-22S. https://doi.org/10.1016/s0002-9343(97)00203-9.

[7] Liu Y, Grimm M, Dai W-T, Hou M-C, Xiao Z-X, Cao Y. CB-Dock: a web server for cavity detection-guided protein-ligand blind docking. Acta Pharmacol Sin 2020;41:138–44. https://doi.org/10.1038/s41401-019-0228-6.

[8] Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 2010;31:455–61. https://doi.org/10.1002/jcc.21334.

[9] Cappuccino JG, Welsh CT. Microbiology: A Laboratory Manual, Global Edition. Pearson Education; 2017.

[10] Forbes BA, Sahm DF, Weissfeld AS, Bailey WR. Bailey & Scott's Diagnostic Microbiology. 12th ed. St. Louis: Elsevier Mosby; 2007.

[11] Willey JM, Sherwood L, Woolverton CJ, others. Prescott's Microbiology. vol. 7. New York: McGraw-Hill; 2011.

[12] Costa SS, Falcão C, Viveiros M, Machado D, Martins M, Melo-Cristino J, et al. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of Staphylococcus aureus. BMC Microbiology 2011;11:241. https://doi.org/10.1186/1471-2180-11-241.

[13] Martins M, McCusker MP, Viveiros M, Couto I, Fanning S, Pagès J-M, et al. A Simple Method for Assessment of MDR Bacteria for Over-Expressed Efflux Pumps. Open Microbiol J 2013;7:72–82. https://doi.org/10.2174/1874285801307010072.

[14] Martins M, Viveiros M, Couto I, Costa SS, Pacheco T, Fanning S, et al. Identification of efflux pump-mediated multidrug-resistant bacteria by the ethidium bromide-agar cartwheel method. In Vivo 2011;25:171–8.

[15] Tripathi N, Goshisht MK, Sahu SK, Arora C. Applications of artificial intelligence to drug design and discovery in the big data era: a comprehensive review. Mol Divers 2021;25:1643–64. https://doi.org/10.1007/s11030-021-10237-z.
[16] BARTHOLOMEW JW, MITTWER T. The Gram stain. Bacteriol Rev 1952;16:1–29. https://doi.org/10.1128/br.16.1.1-29.1952.
[17] Bonnet M, Lagier JC, Raoult D, Khelaifia S. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. New Microbes and New Infections 2020;34:100622. https://doi.org/10.1016/j.nmni.2019.100622.
[18] Abubakar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. J Pharm Bioallied Sci 2020;12:1–10. https://doi.org/10.4103/jpbs.JPBS_175_19.
[19] Mohammedi Z, Atik FA. Impact of solvent extraction type on total polyphenols content and biological activity from Tamarix aphylla (L.) Karst. International Journal of Pharma and Bio Sciences 2011;2:609–15.
[20] Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal 2016;6:71–9. https://doi.org/10.1016/j.jpha.2015.11.005.
[21] Betoni JEC, Mantovani RP, Barbosa LN, Di Stasi LC, Fernandes Junior A. Synergism between plant extract and antimicrobial drugs used on Staphylococcus aureus diseases. Mem Inst Oswaldo Cruz 2006;101:387–90. https://doi.org/10.1590/s0074-02762006000400007.
[22] Kowalska-Krochmal B, Dudek-Wicher R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathogens 2021;10. https://doi.org/10.3390/pathogens10020165.
[23] Rajurkar NS, Hande SM. Estimation of phytochemical content and antioxidant activity of some selected traditional Indian medicinal plants. Indian J Pharm Sci 2011;73:146–51. https://doi.org/10.4103/0250-474x.91574.
[24] Kamalarajan P, Subramani MR, Ganesh M, Valan MF. Phytochemical Investigation of Nilavembu Kudineer Chooranam Ethyl Acetate Extract and Its Ability to Reduce Intracellular Antioxidant Levels in THP-I Cells. European Journal of Medicinal Plants 2020:1–13. https://doi.org/10.9734/ejmp/2019/v30i430187.
[25] Ndam LM, Mih AM, Fongod AGN, Tening AS, Tonjock R, Enang J, et al. Phytochemical screening of the bioactive compounds in twenty (20) Cameroonian medicinal plants. Int J Curr Microbiol App Sci 2014;3:768–78.
[26] Liu Y, Grimm M, Dai W-T, Hou M-C, Xiao Z-X, Cao Y. CB-Dock: a web server for cavity detection-guided protein-ligand blind docking. Acta Pharmacol Sin 2020;41:138–44. https://doi.org/10.1038/s41401-019-0228-6.
[27] Pagadala NS, Syed K, Tuszynski J. Software for molecular docking: a review. Biophys Rev 2017;9:91–102. https://doi.org/10.1007/s12551-016-0247-1.
[28] Zhang S, Wang J, Ahn J. Advances in the Discovery of Efflux Pump Inhibitors as Novel Potentiators to Control Antimicrobial-Resistant Pathogens. Antibiotics 2023;12. https://doi.org/10.3390/antibiotics12091417.
[29] Dulanto Chiang A, Dekker JP. Efflux pump-mediated resistance to new beta lactam antibiotics in multidrug-resistant gram-negative bacteria. Communications Medicine 2024;4:170. https://doi.org/10.1038/s43856-024-00591-y.
[30] Al-Sallami D, Alsultan A, Abbas KH, Clarke SR. Evaluation of efflux pump inhibitory activity of some plant extracts and using them as adjuvants to potentiate the inhibitory activity of some antibiotics against Staphylococcus aureus. Open Vet J 2023;13:42–7. https://doi.org/10.5455/OVJ.2023.v13.i1.5.
[31] Gupta A, Verma A, Katiyar K. Phytochemical-based drug designing against efflux-pump of ESKAPE pathogen to combat multidrug-resistant: an in silico study. Journal of Biomolecular Structure and Dynamics 2025;43:10898–918. https://doi.org/10.1080/07391102.2025.2472401.
[32] Biswas SS, Roy JD. Phytocompounds as potential inhibitors of mycobacterial multidrug efflux pump Rv1258c: an in silico approach. AMB Express 2024;14:25. https://doi.org/10.1186/s13568-024-01673-9.
[33] Samreen, Qais FA, Ahmad I. In silico screening and in vitro validation of phytocompounds as multidrug efflux pump inhibitor against E. coli. J Biomol Struct Dyn 2023;41:2189–201. https://doi.org/10.1080/07391102.2022.2029564.
[34] Ahmad AAM, Abdelgalil SY, Khamis T, Abdelwahab AMO, Atwa DN, Elmowalid GA. Thymoquinone' potent impairment of multidrug-resistant Staphylococcus aureus NorA efflux pump activity. Scientific Reports 2024;14:16483. https://doi.org/10.1038/s41598-024-65991-5.
[35] Lu W-J, Lian Y-W, Chang C-J, Lin H-J, Huang C-Y, Hsu P-H, et al. Screening and Evaluation of Potential Efflux Pump Inhibitors with a Seaweed Compound Diphenylmethane-Scaffold against Drug-Resistant Escherichia coli. Antibiotics 2024;13. https://doi.org/10.3390/antibiotics13070628.