RESEARCH PAPER

Integrating In Silico and In Vitro Studies to develop phytobiotic therapeutics for Aeromonas hydrophila in Aquaculture

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ABSTRACT:
Aeromonas hydrophila is a major bacterial pathogen in aquaculture, known for causing many fish diseases. The rise in antibiotic resistance genes due to antibiotic usage has led to restrictions on antibiotic use, making phytochemicals a promising alternative. In this study, the computational and in-vivo analysis focused on Hemolysin protein as a putative of the Diosgenin against *Aeromonas hydrophila*. In previous literature studies, 376 bioactive compounds were identified from 9 different plants with potent antibacterial activity. The virulence protein of *A.hydrophila* was identified as Hemolysin. Phytocompounds 3-D structures used from PubChem and Molecular docking were performed. Diosgenin showed a good binding affinity with the Hemolysin with a score of -8.7. The most potent inhibitor was carried down for Minimum Inhibitory Concentration (MIC) and Anti-hemolytic assay. MIC indicated Diosgenin has dose-dependent antibacterial activity, and also diosgenin showed potent antibacterial activity against *Aeromonas hydrophila* with the minimum 25 μM concentration, Meanwhile, diosgenin significantly inhibited the hemolytic activity of *A.hydrophila*. The study concludes that Diosgenin, a bioactive phytocompound, effectively inhibits the virulence and hemolytic activity of Aeromonas hydrophila, demonstrating its potential as a natural alternative to antibiotics for managing bacterial infections in aquaculture.

Keywords: Diosgenin, Molecular docking, Hemolysin, Bioactive compounds, Antibacterial Activity.

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Introduction:
Aeromonas hydrophila is a chemo-organ heterotrophic, facultatively anaerobic, freshwater bacteria that infects fish. The gram-negative bacterium Aeromonas hydrophila causes septicaemia, necrotizing fasciitis, and gastroenteritis in fish, which can develop to Motile Aeromonas Septicaemia (MAS) (Cipriano, 1984). Aeromonas species are found in a range of aquatic and environmental environments, such as silt, estuaries, seaweed, sea grass, drinking water, wastewater, and food (Matyar *et al*.,2007). Aeromoniasis has been identified as the most predominant bacterial pathology affecting Indian major carps including Catla catla, Labeo rohita, and Cirrhinus mrigala, as well as exotic carps such as Hypophthalmichthys molitrix, Ctenopharyngodon idella, and Cyprinus carpio throughout the entire year (Sanyal, K.B et al., 2018). A. hydrophila thrives in water at temperatures ranging from 0 to 45 ◦C, with the optimal range being 22-32 ◦C. A. hydrophila infection in fish is a zoonotic disease, meaning it can spread between animals and humans (Dakalov 2006). The pathogenicity of *A. hydrophila* is linked to virulence factors such as hemolysin, cytotoxic enterotoxin, and aerolysin (Jin *et al*., 2020). Hemolysin, produced by the ahh1 gene, is particularly notable for its hemolytic and intestinal toxicity. Efforts to control these infections have included improved sanitation, water management, and the use of antibiotics since the 1990s (Serrano, 2005). However, extensive antibiotic use has led to increased bacterial resistance, posing a public health threat.

As antibiotic resistance escalates, exploring alternative treatments becomes crucial. Natural bioactive compounds or small molecules derived from microbes, marine organisms, plants, and animals are emerging as promising alternatives due to their antibacterial, antioxidant, anti-inflammatory, antiparasitic, and orexis-stimulative properties, which are attributed to compounds like terpenes, flavonoids, and polyphenols (Zhao *et al*., 2015; Hudecová *et al*., 2023). Phyto biotics are bioactive small molecules obtained from plant sources. To screen this type of potential Phyto biotics, a computer-aided drug designing study is one of the efficient and time-saving methods. Molecular docking combined with literature-based screening of these Phyto biotics is a vital computational method in drug discovery that predicts interactions between these molecules and target receptors, aiding in structure-based drug design, virtual screening, and lead optimization by assessing ligand-receptor compatibility (Chaudhary & Tyagi, 2024; Vasant *et al*., 2021).

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And so, this study explores phytobiotic as sustainable alternatives to antibiotics in aquaculture, using molecular docking and literature screening to target bacterial virulence factors and enhance industry health and productivity

Materials and Methods:

Selection of Plants:

Nine different plants were selected for their well-documented antimicrobial properties. This selection was guided by an extensive literature review, which ensured that each plant had a robust history of containing bioactive compounds with proven antimicrobial potential. The chosen plants have been widely recognized in previous research for their effectiveness against a variety of pathogens, making them suitable candidates for this study. The plants included in our study are listed in Table 1.

Preparation of phytochemical compounds:

A total of 376 bioactive compounds from the nine plants were selected from previous literature. 3D structures of the compounds were obtained from the PubChem, in SDF format. Few of the compound structures were available only as 2D structures and therefore, were downloaded as such. Then, the 2D structure of these compounds was converted and optimized as 3D structures by using the ChemDraw Ultra 12 software. Then, all the 3D structures of the compounds were converted into PDB (. pdb) format using the PyMOL v2. 0. 7. Molecular Graphics system (PyMOL: and Schrödinger, LLC; Version 2. 0) and the energy minimization was done by using SwissPDB Viewer V4.0.

Virtual Screening:

Preparation and Optimization of Ligands for Virtual Screening:

Ligands were prepared using AutoDock Tools v1.5.6, where energy minimization was initially conducted using the MMFF94 force field. Subsequently, charges were added, and the rotatable bonds of the ligands were identified. All the ligand files were converted from PDB to the functional PDBQT format to facilitate docking-based virtual screening.

Receptor Preparation for Virtual Screening:

This in-silico study focused on Hemolysin, a target protein to inhibit *Aeromonas hydrophila*. Due to the unavailability of 3D structures in the databases, receptor modelling was carried out using the Swiss Model server with protein sequences obtained from the NCBI database. The modelled 3D structures were validated using the SAVES v6 server. The PDB file of the receptor was prepared for docking by removing water molecules and co-crystallized solvents, adding Kollman charges, and including all hydrogen atoms to ensure accurate computation of partial atomic charges. Grid dimensions for active site marking were set to X=79.443, Y=44.770, Z=66.774, with the centre at X=-4.7792, Y=83.0455, Z=20.7330, encompassing key amino acid residues Val480, His450, and Gly52.

Further, virtual screening was performed using the PyRx software, incorporating the Vina Wizard feature. Pre-prepared ligand and receptor files were loaded into PyRx, where a grid box was assigned around the protein's active site. Docking was executed to identify the highest-scoring configurations, which were subsequently analyzed for further insights (Trott & Olson 2010; Dallakyan & Olson 2015).

Pharmacokinetics study:

The pharmacokinetic characteristics of the potent compounds, including absorption, distribution, metabolism, excretion, and toxicity (ADMET), were analyzed using the pkCSM server (https://biosig.lab.uq.edu.au/pkcsm/). This server evaluates compounds based on their physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness, and toxicity, offering a comprehensive and accessible platform for rapid assessment of various pharmacokinetic parameters. The Lipinski rule of five was applied for ADME analysis. According to Lipinski's rule, a molecule is likely to be drug-like if it meets at least two of the following criteria: a molecular mass of 500 Daltons or less, a log P value of 4.15 or lower, fewer than 10 hydrogen bond acceptors, and no more than five hydrogen bond donors. Additionally, a molar refractivity between 40 and 130 is considered acceptable (Abishad *et al*., 2021).

In-vitro study:

Bacterial preparation:

The bacterial strain used in the study was *Aeromonas hydrophila.* Which was procured from the Vellore Institute of Technology (VIT). The bacteria were cultured in Muller Hinton agar (MHA). Bacterial cultures for antimicrobial testing were prepared by picking colonies from 24-h-old Muller Hinton agar plates and it was suspended in an appropriate medium(5 mL). Cultures were grown anaerobically overnight and continuously shaken at 60 rpm at 30 °C.

Compound:

Diosgenin was purchased from Sisco Research Ltd (SRL) With a purity of 95 % (CAS: 512-04-9). Molecular Weight: 414.62, Crystalline powder.

The antibacterial colony-forming unit (CFU) assay:

The antibacterial colony-forming unit (CFU) assay involves preparing an *Aeromonas hydrophila* culture by inoculating a single colony into the nutrient broth and incubating at 37°C overnight. The culture suspension was then adjusted to a 0.5 McFarland scale and divided into aliquots for treatment with diosgenin and controls. After incubation, serial dilutions of the treated and control suspensions are performed, and 100 µL of each dilution is plated onto nutrient agar plates. These plates are incubated for 24 hours, after which the colonies (CFUs) are counted. Plates with 30-300 colonies are used for accurate counting, and the CFU per mL is calculated using the formula:

CFU/mL = (Number of Colonies x Dilution Factor) / Volume Plated.

The results from treated and control groups are compared to evaluate the antibacterial efficacy of the diosgenin, with statistical analysis performed to determine significance. All steps are conducted under sterile conditions, and the assay is performed in triplicate (Cong, S *et al*.,2020; Balouiri *et al*., 2016)

Minimum Inhibitory concentration:

To perform a Minimum Inhibitory Concentration (MIC) assay, *Aeromonas hydrophila* was inoculated in Mueller-Hinton broth (MHB) and incubated overnight at 37°C, then adjusted to 0.5 McFarland standard. In a 96-well microplate, 100 µL of MHB is dispensed into each well, followed by a two-fold serial dilution of diosgenin. The standardized bacterial suspension (100 µL) is added to each well, resulting in a final concentration of approximately 5×10^5 CFU/mL. Control wells include MHB only (negative control) and streptomycin antibiotic (CAS No. 3810-74-0) as positive control. The microplate is sealed and incubated at 37°C for 16-20 hours. Bacterial growth is assessed by measuring optical density at 600 nm or visually inspecting for turbidity. The MIC is determined as the lowest concentration of diosgenin that inhibits visible bacterial growth. The assay is performed in triplicate and results are compared to standard breakpoints to evaluate the effectiveness of diosgenin. All procedures are conducted following laboratory safety protocols.

Hemolytic assay:

The effect of Diosgenin on hemolytic activity was determined by spectrophotometric measurement of haemoglobin released from erythrocytes exposed to the bacterial culture supernatant. Heparinized blood samples were collected from healthy volunteers. Two millilitres of whole blood were added to 4 mL of 0.9% saline and centrifuged at 10,000 rpm for 5 minutes to isolate red blood cells (RBCs). The RBCs were then washed five times with 10 mL of D-PBS and finally diluted to 20 mL with 0.9% saline. An overnight culture of *Aeromonas hydrophila* with and without diosgenin at different concentrations (25 µL, 50 µL, and 100 µL) was incubated at 37°C for 3 hours. Subsequently, the same volume of the previously prepared 0.2% erythrocyte suspension and the treated and non-treated samples were added to 15 mL Falcon tubes and incubated for 2 hours at 36 ± 1°C. The tubes were centrifuged at 10,000 rpm for 5 minutes, and 100 µL of the supernatant was added to a 96-well plate to measure absorbance at 540 nm. All steps were conducted under sterile conditions, and performed in triplicate. The percentage of hemolysis was calculated using the formula:

$$\% \text{ of Hemolysis} = \left\{ \frac{\text{Absorbance of Sample} - \text{Absorbance of Negative Control}}{\text{Absorbance of Positive Control} - \text{Absorbance of Negative Control}} \right\} \times 100$$

Results

In silico analysis of 376 compounds was screened against the hemolysin protein of *Aeromonas hydrophila* using Pyrx software. Nine unique poses were obtained. Among those with the lowest binding score (highest binding affinity), lead compounds were selected.

Among all the 5 compounds. Diosgenin, which shows good binding affinity, was chosen as the drug candidate for the study. The molecule diosgenin has a molecular weight of 414.6 g/mol and demonstrated a significant binding energy of -8.4 kcal/mol with hemolysin (Figure 1.). It forms different ligand–protein interactions which include conventional and hydrogen bonding with residue alanine residue at position 351 (Ala 351), with a bond length of 2.92 Å. It forms hydrophobic interaction with lysine 157 (Lys 157), tyrosine 125 (Tyr 125), alanine 248 (Ala 248), valine 153 (Val 153), asparagine 415 (Asn 415), and asparagine 353 (Asn 353), alkyl and p-alkyl interaction with Tyr 125, Lys 157, Ala248, val413, Ala351 (Figure 2.).

Pharmacokinetics study:

Diosgenin demonstrated a favourable pharmacokinetic and toxicity profile characterized by high intestinal absorption exceeding 30%, and excellent plasma binding properties with 97.743% of the compound bound to plasma proteins and only 1.872% remaining unbound, along with a volume of distribution of 1.695 L/kg. Metabolic analysis revealed that diosgenin did not inhibit any major CYP450 enzymes, including CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, indicating minimal interaction with common metabolic pathways. Toxicity evaluations predicted no hepatotoxicity, AMES toxicity, or skin sensitization associated with diosgenin. Additionally, diosgenin exhibited a high clearance rate of 23.332 mL/min/kg, ensuring efficient excretion. Furthermore, diosgenin showed promising permeability across the blood-brain barrier (BBB), suggesting potential for central nervous system effects without significant safety concerns. Diosgenin has one H-bond donor and three H-bond acceptors, giving it a molecular weight of 414.62 g/mol which is approved by Lipinski's rule of five. These properties collectively highlight diosgenin's potential as a safe and effective therapeutic compound in the field of fish disease management.

Invitro study

Antibacterial Activity: Diosgenin exhibited potent antibacterial properties against *Aeromonas hydrophila*, as evidenced by a significant reduction in bacterial colonies with increasing concentrations of diosgenin. At concentrations of 25, 50, and 100 µM, there was a clear dose-dependent decrease in the number of colonies, demonstrating that higher diosgenin concentrations were more effective in reducing bacterial growth (Figure 3.).

Minimum Inhibitory Concentration (MIC): The MIC of diosgenin, the lowest concentration needed to inhibit bacterial growth, was identified at 25 µM. This indicates that diosgenin at this concentration is effective in

preventing the proliferation of *A. hydrophila*. Streptomycin used as a positive control, showed strong inhibition at all tested concentrations, supporting the results of diosgenin's antibacterial effectiveness and validating the assay's reliability.

Hemolytic Assay: Diosgenin also demonstrated a significant ability to inhibit the hemolytic activity of *A. hydrophila*. At a 25 µl concentration, diosgenin caused moderate hemolysis with optical density (OD540) values similar to the positive control, which indicates an initial level of effectiveness. As the concentration increased to 50 and 100 µM, there was a marked reduction in hemolysis, highlighting diosgenin's potential to counteract the damaging effects of bacterial hemolysin toxins on erythrocytes (Figure 4.).

These findings collectively underscore the therapeutic potential of diosgenin as an effective antibacterial agent and its capability to mitigate bacterial virulence factors. The dose-dependent reduction in bacterial colonies and hemolytic activity with increasing diosgenin concentrations suggests its promising application in treating bacterial infections, particularly those caused by *Aeromonas hydrophila*. The research emphasizes the importance of further exploring diosgenin's mechanism of action and its potential integration into antimicrobial therapies.

Discussion:

The present study elucidates the antibacterial and hemolytic inhibitory properties of diosgenin against *Aeromonas hydrophila*, a clinically significant pathogen associated with both human infections and aquaculture-related diseases (Cipriano, 1984).

Diosgenin has gained prominence due to its potential to treat various deadly conditions, including diabetes mellitus, atherosclerosis, osteoporosis, skin conditions, cardiovascular diseases, and cancer (Kalailingam *et al*., 2015; Hua *et al*., 2016; Semwal *et al*., 2022). Also known as 25R-spirost-5-en-3β-ol, diosgenin is a dioscin hydrolysate found in the rootstock of yams (Dioscorea) and is widely distributed as a glucoside in natural plants. It is a steroidal sapogenin present in various plants, such as Costus speciosus and Dioscorea nipponica Makino (Patel *et al*., 2012).

Despite its therapeutic potential, only a limited number of studies have investigated the antibacterial properties of diosgenin. Recent research has shown that diosgenin exhibits significant antibacterial activity against *Aeromonas hydrophila*, with a dose-dependent reduction in bacterial colonies observed at concentrations of 25, 50, and 100 µL (Semwal *et al*., 2022). The minimum inhibitory concentration (MIC) of diosgenin was determined to be 25 µL, which effectively inhibited bacterial growth, comparable to the positive control, streptomycin (Semwal *et al*., 2022).

Additionally, diosgenin demonstrated a notable ability to inhibit the hemolytic activity of *A. hydrophila*. At a concentration of 25 µl, diosgenin showed moderate hemolysis, but higher concentrations (50 and 100 µl) reduced hemolysis up to 7%, indicating its capacity to mitigate bacterial hemolytic activity (Semwal *et al*., 2022). Diosgenin also displayed favourable pharmacokinetics and toxicity profiles, including high intestinal absorption, excellent plasma binding properties, no inhibitory action against CYP450 enzymes, and no predicted hepatotoxicity, AMES toxicity, or skin sensitization (Semwal *et al*., 2022). Furthermore, diosgenin exhibited promising permeability across the blood-brain barrier (BBB), suggesting potential central nervous system effects without significant safety concerns. These findings collectively highlight diosgenin's potential as a safe and effective therapeutic compound, warranting further exploration of its mechanisms and applications in antimicrobial therapies (Semwal *et al*., 2022).

In this study, 376 phyto-compounds from Nine medicinal plants, having good antibacterial properties were subjected to Molecular docking studies. Hemolysin a virulence protein of *Aeromonas hydrophila* was selected as the receptor complex. Auto dock vina program in PyRx was used (Trott, O., & Olson, A. 2009). Among the 376 phyto-compounds screened, the top 5 compounds that have efficient binding affinities with the active site of hemolysin were identified. Dioscin, Diosgenin, and methyl protogracillin showed binding affinity of -10.1, -8.7, and -9 respectively from the same plant *Costus speciosus*. Taraxasterone showing binding affinity -8.8 is from the plant Sida acuta Burm.f. Kaempferitrin showing the binding affinity -9.1 was from the plant cleome viscosa. Cong, S, *et al*. 2020 investigated the antibacterial efficacy of diosgenin against *Porphyromonas gingivalis* and *Prevotella intermedia*. And do Socorro Costa, M *et al*. investigated the antibacterial and inhibitory activities of diosgenin on NorA and MepA efflux pumps from *Staphylococcus aureus*. They determined that diosgenin had antibacterial properties against this bacterium.

Our study expands on this body of information by demonstrating diosgenin's antibacterial activity against *Aeromonas hydrophila*, a pathogen of great importance in both clinical and aquaculture settings. In molecular docking studies, 9 plants were chosen and 376 compounds were tested, with the top 5 scoring ligands being diosgenin, which demonstrated a high binding affinity and hydrogen bond interactions. Even though the other four compounds have a high binding affinity, diosgenin was chosen for further study. The selection of diosgenin was further validated by its low cost and the significant body of previous research supporting its biological actions.

While diosgenin's antibacterial properties have been explored in other contexts, such as against *Porphyromonas gingivalis* and *Staphylococcus aureus* (Cong *et al*., 2020; Do Socorro Costa *et al*., 2020), this study is among the first to demonstrate its activity against *Aeromonas hydrophila* and this shows the novelty of our study with our research findings. Given the prevalence of this pathogen in aquaculture and clinical infections, diosgenin offers a natural, cost-effective alternative to synthetic antibiotics. The findings emphasize diosgenin's dual role as an antibacterial and anti-hemolytic agent. Further research should explore its mechanism of action in disrupting hemolysin activity and evaluate its efficacy in vivo. Future studies should focus on scaling up in-vivo trials to evaluate the safety and efficacy of Diosgenin in

diverse aquaculture settings, exploring its mechanism of action at the molecular level, and assessing its potential synergistic effects with other phytochemicals or probiotics for comprehensive disease management.

Conclusion

This study reveals diosgenin as a promising lead phytobiotic against *Aeromonas hydrophila*, indicating its potential for medicinal use. Diosgenin had a high binding affinity to the target receptors, demonstrating a substantial interaction with bacterial proteins important for pathogenesis. The diosgenin's favourable pharmacokinetic qualities increase its viability as a therapeutic agent and suggest minimal risk of adverse drug interactions. The antibacterial activity of diosgenin was confirmed by a significant reduction in bacterial colony counts, indicating a dose-dependent inhibitory effect. Furthermore, diosgenin effectively suppressed *A. hydrophila*'s hemolytic activity, minimizing erythrocyte lysis and implying its anti-toxin potential against the bacterial toxin. Collectively, these findings support the advancement of diosgenin as a viable candidate for the treatment of bacterial infections, particularly those caused by *A. hydrophila*, and the necessity for additional in vivo and other laboratory, preclinical, and clinical investigations to fully explore its therapeutic potential.

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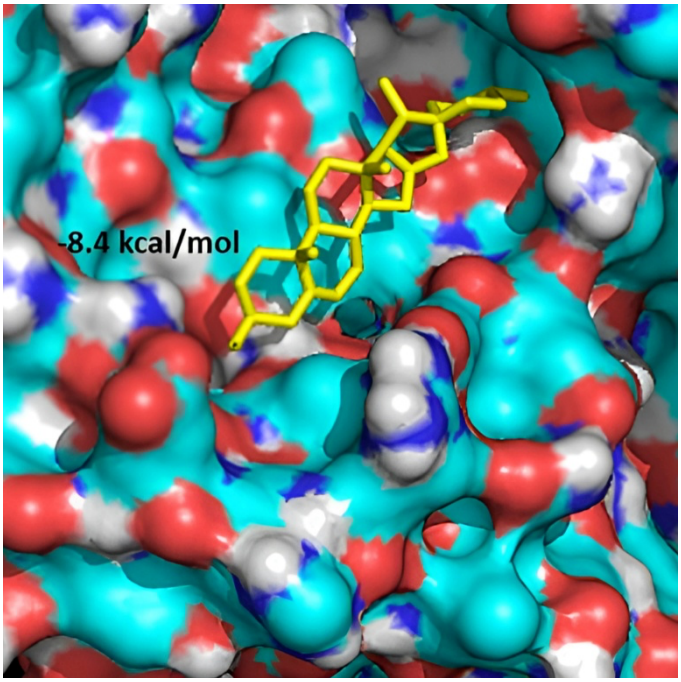

Figure 1. Diosgenin docked in the active site of the hemolysin protein of *A. hydrophila*

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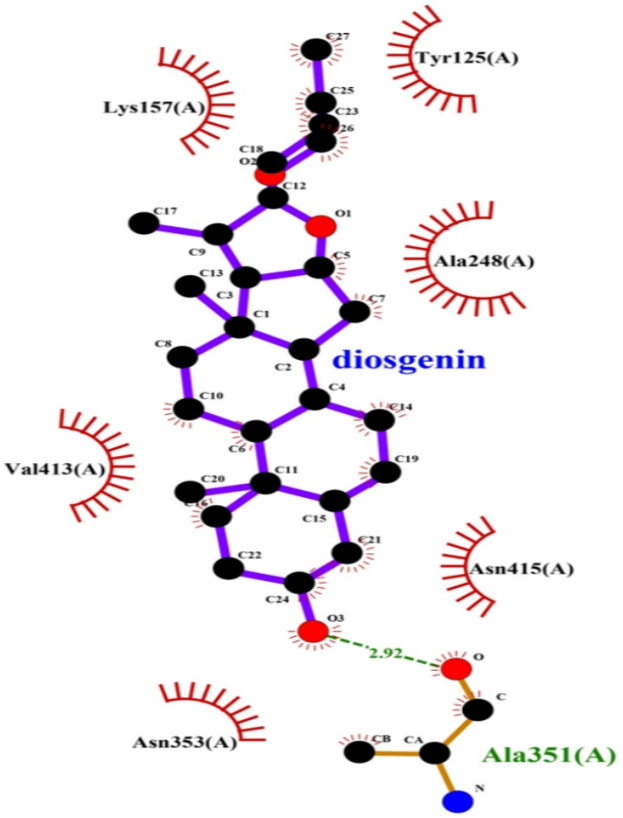

Figure 2. 2D interaction plot of Diosgenin interacted with the amino acids in the active site of hemolysin protein

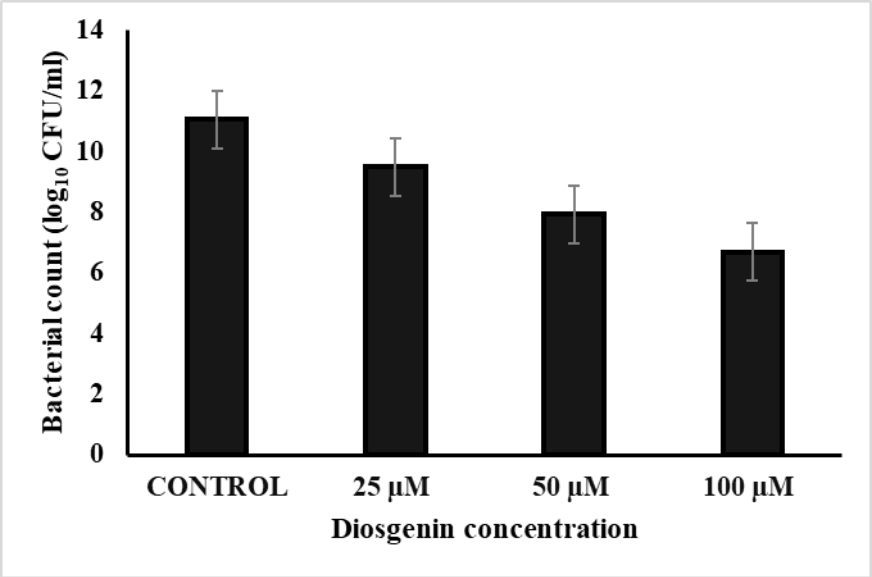

Figure 3. Antibacterial efficacy of Diosgenin exhibited by reduced CFU of *A. hydrophila*

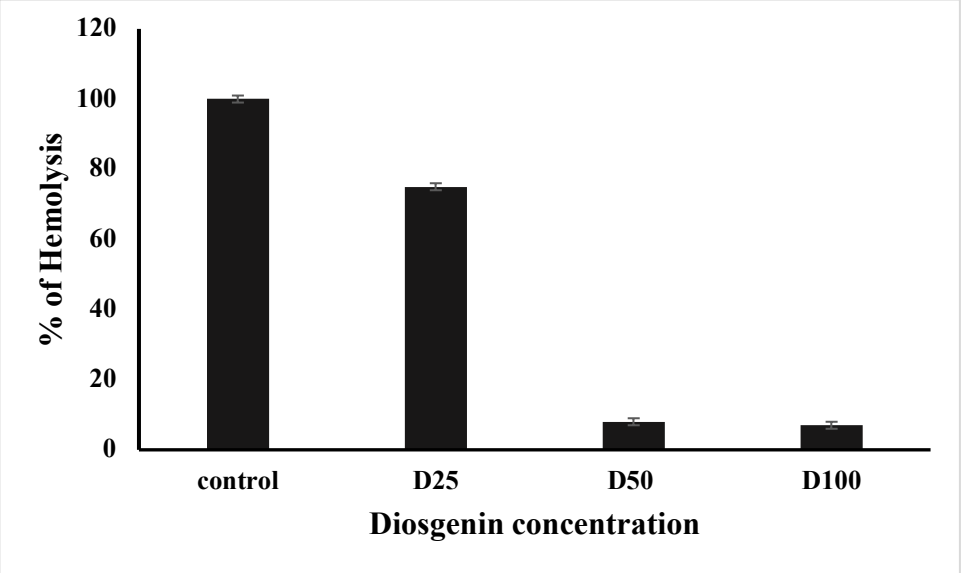

Figure 4. Anti-toxin efficacy of Diosgenin exhibited by reducing the hemolytic activity of hemolysin of *A. hydrophila*

s.no	Scientific name	Common name	uses	References
1	*Tragia involucrate*	Indian stinging nettle	Antimicrobial, Anti-inflammatory, Antioxidant	Solanki R.,2011
2	*Costus speciosus*	crêpe ginger	Anti cancer , Anti-inflammatory, Antidiabetic, Hepatoprotective, Hypolipidemic, Adaptogenic, Antimicrobial	El-Far, A., et al ., 2018
3	*Molluga cerviana*	thread stem carpetweed	anti-inflammatory, antioxidant activity, spermicidal activity	Aglin, A. A. (2018)
4	*Plectranthus Vettiveroides*	Vetiver	Antibacterial, genitourinary diseases, intrinsic haemorrhage	Nisheeda, B. A.,et al., 2016
5	*Cleome viscosa*	wild or dog mustard	Anthelmintic, Antiseptic, carminative, Antimicrobial Antiscorbutic, Sudorific, febrifuge, and Cardiac stimulant	Mali, R. G. (2010)
6	*Clerodendrum phlomidis*	bleeding-heart	Analgesic, Anti diarrhoeal, Anti plasmodial, Antiinflammatory, hypoglycemic, Antifungal, nematicidal, Anti-amnestic and Anti-arthritic	MK, M. M. R ., & Mishra, S. H. (2010)
7	*Sida acuta brum.f*	Broom weed	Anthelmintic, Antiemetic, demulcent, diuretic, Aphrodisiac, stomachic, Antimicrobial, antipyretic and wound healing properties	Senthilkumar, R. P et l., 2018
8	*Trichosanthes cucumernia*	Snake Gourd	Analgesic and antibacterial	Fathima, M. Z et al.,2017
9	*Arundo donax*	Giant reed	Anti-bacterial, Anti-oxidant, Anti-proliferative, Anti-spasmolytic, and also used to treat helminthic	Kumar, P., et al.,2021

Table 1. Pharmacological potential of selected ethnomedicinal plants with documented therapeutic uses