

Antibacterial Activity of Herbal *Plantago major* and *Plantago lanceolata* extracts on *Pseudomonas aeruginosa* with emphasis on Exotoxin A gene expression and Bioinformatics approach

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Abstract

Pseudomonas aeruginosa contamination could be a significant cause of dismallness in burns patients. To detect the antibacterial potentials of *Plantago lanceolata* and *Plantago major* extracts against *Pseudomonas aeruginosa* and assess their potential to control the *toxA* gene expression with a bioinformatics approach. The antibacterial sensitivity test, MIC, and MBC were performed. The expression value of *toxA* was evaluated using a qPCR assay. A molecular dynamics simulation and toxicity risk assessment were done. Results indicated a remarkable antibacterial activity of the crude extracts compared to other fractionated plant extracts. The mean zone of inhibition ranges up to 16mm (*P. major*, 1000 mg/mL) and 20 mm (*P. lanceolata*, 1000 mg/ml) against *P. aeruginosa*. Water extract showed the inhibition zone, while the Petroleum ether and chloroform did not show any inhibition activity. *Tox-A* gene expression value had been significantly ($P < 0.05$) decreased at the most concentrations of both plants. Molecular docking and toxicity risk assessment revealed that luteolin ligand (LD50: 5000 mg/kg) with a lower toxicity dose could be a potential drug for treating *P. aeruginosa* infections. Findings revealed that the crude extract of both plants has possible antibacterial activity and could be considered against *P. aeruginosa* pathogenicity. Furthermore, Luteolin might be a helpful drug for treating *P. aeruginosa* infections.

Introduction

Over the most recent couple of years, most pathogenic organisms created protection from numerous antibiotics, a significant danger to human well-being [1]. *Pseudomonas aeruginosa*, an audacious and opportunistic pathogen, is a critical reason for medical services-related diseases, being especially hazardous in intensive care units [2]. Its infections are related to high morbidity and mortality in numerous gatherings, incorporating burns patients and people with medical services related to pneumonia, chronic obstructive pulmonary disease (COPD), or cystic fibrosis (CF)[3–7]. Burn wound infection due to this bacterium triggers a significant challenge in Burn wound sepsis and even graft loss in patients[8]. In Gram-negative, aerobic bacillus *Pseudomonas aeruginosa*, it has been revealed that the most toxic virulence factor is the exotoxin A (ADP-ribosyl transferase) is secreted by the T2SS secretion system [9], which contributes to its pathogenesis by secreting to the extracellular surroundings and binding to the host cells which gives this pathogen remarkable plasticity [9–12]. *Pseudomonas aeruginosa* is resistant to major classes of antibiotics [13]. This phenomenon has posed a significant challenge for conventional therapeutic approaches[14]. There has been considerable interest in using plants and their components to dispose of pathogenic microorganisms due to expanding antibiotic resistance [15].

Plantago is one of the oldest medicinal plants that has been broadly used in the traditional medicine. It may grow wild across Asia, Europe, America, and practically all of Iran. *Plantago* species have been discovered in 275 locations across the world. *Plantago major* and *Plantago lanceolata* are perennial medicinal herbs from the Plantaginaceae family used for centuries to treat numerous diseases, particularly digestive organs, reproduction system, circulation, burn, and inflammatory skin diseases. *P. major* is a perennial species that grows from a short, thick taproot. It has broad oval dark green basal leaves with green to small white flowers borne in a dense spike[16–18]. There are various phytochemical compounds in the subgenus of *Plantago*. A number of them, such as alkaloids, caffeic acid derivatives, coumarins, fats and oils, flavonoids, iridoids, mucilage, polysaccharides, sterols, and volatile substances, have been referred to in various studies [17, 19].

The opportunistic bacteria *Pseudomonas aeruginosa* secretes exotoxin A (PE), a highly toxic protein. PE becomes a selective agent for deleting specific cell populations when combined with various artificial targeting components such as receptor ligands and antibody fragments [20]. PE's modular structure and matching mode of action makes it amenable to a wide range of changes that can turn its significant cytotoxicity into a therapeutic function.

In the current research, to minimize the *Pseudomonas aeruginosa* resistance to traditional antibiotics, *Plantago major* and *Plantago lanceolata* medicinal herb plants were applied to determine plant extracts potentials against antimicrobial activity and Exotoxin A gene expression. Furthermore, molecular dynamics simulations and toxicity risk assessments of compounds were performed to evaluate their potential drug activity.

Materials And Methods

Patients - clinical samples

Four isolates, A, B, C, and D of *Pseudomonas aeruginosa*, were recovered from burn wound infection in patients in the dedicated burns unit-Shahid Motahari hospital-Tehran. The samples were collected in Nutrient agar containing 0.5% Peptone, 0.3% beef extract, 1.5% agar, and 0.5% Sodium Chloride (37 °C / 24 h) and then incubated aerobically at 37 °C for overnight in a Mueller-Hinton agar containing 2.0 g beef extract, 17.5 g casein hydrolysate, 1.5 g starch and, 17.0 g agar. *Pseudomonas aeruginosa* strain ATCC 9027 was selected as a standard (STD) control (RRID:WB-STRAIN:WBstrain00041978).

Bacterial Identification

Diagnostic test of bacteria was performed using Gram staining and biochemical tests, including catalase, oxidase, glucose fermentation test, pigment formation, and growth at 42 °C in cetrimide agar and arginine dehydrogenase.

Plants collection, identification, and preparation of plant extract

The leaves of *Plantago major* and *Plantago lanceolata* were collected from Tehran and were identified by the Research Institute of Forests and Rangelands. Then 100 grams of the leaves were separately dried in the shade, pulverized by a mechanical grinder, and stored in airtight glass containers in the dark until extraction. Ethanolic extraction (50%) was performed through the Maceration method. 30 mL of distilled water was added to 6 grams of plant extracts and dissolved well at 40 °C. Then, the volume of the solution was increased to 250 mL in a decanter funnel. Then, 200 mL of Petroleum ether was added to it, and after shaking, the petroleum phase was separated. To the remaining aqueous phase was added 100 mL of chloroform, because of which the chloroform

phase was separated from the aqueous phase. As a result of this method, three fractions, including water, chloroform, and petroleum, were isolated and prepared [21,22].

Extraction of flavones apigenin and Luteolin using Isocratic HPLC

Flavones, Apigenin, and luteolin tests were arranged by dissolving the extract in acetonitrile and diluting it with 50% acetonitrile. This solution was analyzed by reverse-phase chromatography on an Altima C8 column with isocratic elution of acetonitrile and orthophosphoric acid (1:1 v/v) with a flow rate of 1 mL.min⁻¹, a column temperature of 33 °C, and UV location at 204 nm[22].

Susceptibility test

Antibacterial susceptibility of *P. major* and *P. lanceolata* extract materials was done using the agar disc diffusion method described by Rajesh Kumar *et al.* [23,24]. McFarland 0.5 turbidity standard was prepared (0.5 mL of a 1.175% w/v) barium chloride dehydrates (BaCl₂·2H₂O) solution + 99.5 mL of 1% (v/v) sulfuric acid). The suspension turbidity was made until comparable to 0.5 McFarland standards; then, from the suspension, a volume of 100 µL was added to solidified Muller-Hinton agar plate. Extractions and their fractionated materials were diluted with 800 µL Dw and 200 µL DMSO. For dilution, first, a concentration of 1 gr/mL of the extract was prepared, and then concentrations of 500, 250, 125, and 62.5 mg/mL were made from it, and the results were recorded. After 24 hours, the inhibition zone of all plates was measured with MIP (Microscopic image processing) software. For increasingly correct examination, the micro-dilution method performed MIC and MBC tests. Which was performed according to the CLSI standard, and the results were recorded. In this test, only a total extract was used. MIC is the minimum inhibitory concentration of antimicrobial agents, and minimum bactericidal concentration (MBC) is the least concentration of plant extracts required to kill bacteria evaluated by micro dilutions or broth dilution methods [24, 25]. For this purpose, bacteria streaked onto Müller-Hinton Agar plates without inhibitor and then incubated for 24h at 37 °C. Colonies were transferred to a glass with 5 ml of MH-Broth medium and incubated for 24 h at 37°C. At the end of the experiment, the growth of bacteria was measured at 450 nm using an ELISA reader: Milton Roy-Spectronic21D- USA. Every plate was used three times (triplicates) to exclude any errors.

Molecular Docking simulations

To investigate the model of the Luteolin and Apigenin coupling with the active site of Exotoxin-A, the chemical structures of components were obtained from PubChem and RCSB Protein data bank. Luteolin (PubChem CID: 135952), Apigenin (PubChem CID: 79730), and using RCSBPDB, a crystal structure of *Pseudomonas aeruginosa* exotoxin A (PDB ID: 1LKQ, DOI: 10.2210/pdb1LKQ/pdb) was downloaded to perform molecular modeling and dynamics simulations. Site-specific molecular docking study was conducted using the Auto Dock Vina program (using a Lamarckian genetic algorithm), and the energy minimization was done using the Hyper-Chem software package. Then the results were analyzed utilizing the Molegro Virtual Docking software [26]. Furthermore, the toxicity risk assessment of compounds was performed using the OSIRIS program. The chemical structure characteristics of ligands and proteins are shown in Table1.

Quantitative PCR- Exotoxin-A gene expression study

RNA extraction and cDNA preparation

Following extraction of bacterial RNA and converting it to cDNAs using total RNA extraction commercial kit and easy cDNA synthesis kits respectively purchased from Parstous biotechnology-Iran according to manufactures instructions, a quantitative polymerase chain reaction (qPCR) was used for detection of exotoxin-A expression value [27,28].

Q-PCR assay

A quantitative PCR assay was conducted in a total volume of 20 µL in 0.5 mL microtubes using Rotor-Gene Q's real-time PCR detection system. The primers were designed using primer3 input version4 online software (<http://bioinfo.ut.ee/primer3-0.4.0/>), and the efficacy of primers was assessed using Beacon Designer and Mfold software. In this research study, the primer sequences were served as F-5'TTC GTG GAT GAA CAC CTT GA 3', R-5'TGC TGC ACT ACT CCA TGG TC3' for Exotoxin A gene, and F-5'CTG GAG AGA CTA AGC CCT CC 3', R-5'ATT ACT GAC GCT GAT TGT GC3' for 16s rRNA as the housekeeping gene. PCR- Reaction was performed (using SYBR Green real-time PCR Master kit- Parstous-Iran) in a 20 µL solution having 10µL of CYBR Green Master mix (2X), 2 µL of each primer (10 pmols), and 1 µL of cDNA and 5 µL of distilled water. Amplicon condition was conducted at 94 °C/10 min for the first denaturation, followed by 32 cycles for 95 °C/15sec denaturation, 60 °C/30sec annealing, and 72 °C/60-sec extension. This was followed by a melting curve analysis for each PCR amplicon, and gene expression was normalized to 16s rRNA as an internal control. The data were analyzed by the $\Delta\Delta C_t$ method. Fold changes of Exotoxin in the equation $2^{-\Delta\Delta C_t}$ calculated a gene [29]. All reactions were done in triplicates.

Statistical Analysis

Statistical analysis of the effect of Bacteria and concentration on gene expression was performed by the 2-way ANOVA method. Furthermore, the ANOVA test calculated the significant difference in expression observed between the groups. Gene expression differences were computed using Gene x6 software, statistical analyzes were performed using SPSS 21 software (RRID:SCR_014598), and graphs were plotted with graph pad prism 8 (RRID:SCR_002798).

Results

HPLC analysis

Based on Isocratic HPLC analysis, the pick of apigenin-7-glucoside and Luteolin were obtained at 8.46 and 24.42 minutes for *Plantago major* and *Plantago lanceolata* (Fig. 1). Apigenin and luteolin have been in *P. major* 0.156%, 0.143% and in *P. lanceolata* 0.02%, 0.062%, respectively.

Antibacterial activity

Antibacterial susceptibility test results of *P. major* and *P. lanceolata* extract materials are shown in Fig 2 and Table2. The results of inhibition zones varied depending on the type of plant extracts and plant species. Furthermore, both Plants' Petroleum ether and chloroform fractionation did not show any significant activity against *Pseudomonas aeruginosa*.

MBC and MIC

The Minimum Bactericidal Concentration and the Minimum Inhibitory Concentration values of *P. major* and *P. lanceolata* extracts materials on pseudomonas isolates are shown in Table3. These results indicated that *P. lanceolata* inhibited the growth of Pseudomonas isolates successfully. Among the plant species tested, *P. major* had a higher MBC (250 mg/mL) and MIC (125 mg/mL) than *P. lanceolata*. Among different strains, strain B was more resistant to growth inhibition, and strain A was more resistant to the lethality of *P. lanceolata* whole leaf extract than other strains and was within the standard strain. Equality of MIC and MBC of *P. major* leaf extract in all strains except strain 2 indicates that this extract will kill bacteria without inhibiting their growth.

Molecular modeling of Luteolin and Apigenin interactions with Exotoxin-A protein

Molecular modeling was performed against Exotoxin-A crystal to predict and compare the inhibitory effects of Luteolin and Apigenin, and the average results were computed. The parameters calculated for the target (EXO-A) demonstrated that the average binding energy and inhibition constant (Ki) was -8 kcal/mol (clRMSD: 0, binding affinity: -8) for Apigenin and -9.71 kcal/mol (clRMSD: 0, binding affinity: -9.71) for Luteolin (Table 4). Site-specific docking revealed that Luteolin (CID135952, clRMSD:0, binding affinity: -9.71) had more relationship to inhibiting exotoxin-A protein. In the ADMET analysis study, the results from the fafdrug4 server show that the Apigenin ligand (CID79730) is the accepted ligand for the drug. Still, ligand CID135952 is out of the filters due to its high molecular weight and too many H-bond Acceptors, which have been rejected in this respect. On the other hand, the results of the protox2 server, which was performed to evaluate the toxicity of ligands in terms of LD50, show that the Apigenin ligand has a lower concentration (LD50: 3919 mg/kg) and, therefore higher dose toxicity than the luteolin ligand (LD50: 5000 mg/kg). A schematic presentation of molecular modeling inhibition of ligands and Exotoxin-A protein and amino acid residue interactions has been shown in Fig3.

Quantitative PCR results

A Gene expression study was performed on three different concentrations of more MIC, including 62.5, 125, and 250 mg/mL of *P. major* extracts and 15.62, 31.25, and 62.5 mg/mL of *P. lanceolata* extract. Fig 2 clearly shows that the expression level of pathogenic genes exotoxin A (*toxA*) declined after exposure to *Pseudomonas aeruginosa* with *P. major* extract materials. The results show that compared to the control group, the *toxA* gene significantly ($P < 0.05$) downregulated in all isolates at all concentrations of *P. major* extracts except for C-isolate; the gene was in an overexpression pattern (Fold =29.75376, $P = 0.000$) in 250 mg/mL. In contrast, the *toxA* gene significantly ($P < 0.05$) downregulated in all isolates at all concentrations of *P. lanceolata* extracts exception of A and C-isolates; the gene was in an overexpression pattern at 62.5 mg/mL and 250 mg/mL of plant extract concentrations respectively when the *Pseudomonas aeruginosa* exposed to *P. lanceolata* (Fig. 4). A comparison diagram of *toxA* gene expression in different strains has been shown in Fig.5. *ToxA* gene expression had significantly decreased at most concentrations. In strain A, gene expression increased at a 15.62 mg/mL concentration of *P. lanceolata* extract. In strain C, a significant increase in *toxA* gene expression was observed under the influence of the highest concentration of extracts of the two plants (Fig. 6).

Discussion

Pseudomonas aeruginosa infection is a significant cause of morbidity in burns patients. In burns due to skin destruction as the first line of the body defense system, a patient is very susceptible to infections [8]. It is well known that the most important virulence factor of *P. aeruginosa* is exotoxin A which plays a role in biofilm formation and pathogenicity and therefore is considered a critical factor in antimicrobial resistance [30]. PE is a three-domain bacterial toxin that kills mammalian cells by obtaining access to the cytosol (increasing entrance) and inactivating protein synthesis. Binding to a surface receptor, internalization through coated pits and endosomes, proteolytic processing, disulfide bond reduction, and lastly, translocation of an enzymatically active C-terminal fragment to the cytosol are all steps in the toxin entry route[31]. Exotoxin A is exceedingly deadly, causing histological abnormalities in the liver, inducing hepatocyte death, and increasing the production of many proinflammatory cytokines, according to Fogle et al. [32]. A research study has revealed a significant association between exotoxin A with antimicrobial resistance in *P. aeruginosa* [33]. Because of the extreme resistance of *P. aeruginosa* to antibiotics, finding complementary treatment is required. In this research, antimicrobial activity and Q-PCR technique have been used to study *P. major*, and *P. lanceolata* extracts on the *toxA* gene expression.

Both *P. major* and *P. lanceolata* are applied for different purposes in biological activities and traditional medicine worldwide. *P. major* is used to treat a variety of wounds and skin conditions, including deep wounds, purulent wounds, chronic and progressive wounds, malignant wounds, fire burns, erysipelas, progressive blisters, pruritus, irritating urticarial, and fistulas, by sprinkling the plant powder on the wound or its plaster with salt, or separately[34]. Further research has shown that *P. major* and *P. lanceolata* have antibiotic activity against some gram-negative, gram-positive bacteria and fungi [14, 18]. A research study conducted by Kassaw *et al.*, examined the therapeutic potential antibacterial effects of *P. lanceolata* ethanol extract compared to chloroform extract on *Salmonella typhimurium* (standard pathogenic bacteria) [18]. Monjd *et al.* studied the antibacterial effects of *Plantago major* compared with *Ceratonja siliqua*, which is used in Iraqi folk medicine as an antidiarrheal against gram-positive and gram-negative pathogens. Their results revealed more effectiveness of *P. major* against tested bacteria[35]. In the same research conducted by Koohsari *et al.*, *P. major* ethanol extract had the most antibacterial activity. Their study

showed that gram-positive bacteria such as *Staphylococcus epidermidis* and *Staphylococcus aureus* were more sensitive than gram-negative bacteria[14]. Our results showed a remarkable antibacterial activity of the crude extracts (total = 1000 mg) of these plants, and the largest diameter of the inhibition zone was observed close to that of positive control. This finding was in agreement with other studies [14, 18]. As a result, in our research, Petroleum ether and chloroform extract did not show an inhibition zone against *P. aeruginosa*. Najafian *et al.*, indicated that an aqueous extract of *P. major* ethnophenol, diathiapentene, naphthalene, and glycerin was found. In methanol extract, a group of diglycerol and glycol contain organic acids, such as fumaric acid, syringic acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid, p-coumaric acid, Gentisic acid, traces of salicylic acid, benzoic acid and cinnamic acid were found[33].

We also studied the expression pattern of Exotoxin A gene (*toxA*) value after exposure of *P. aeruginosa* with *P. lanceolata* and *P. major* in different concentrations of extracts materials. To the best of our knowledge, this is the first report on the effects of *P. lanceolata* and *P. major* extracts on the Exotoxin-A expression pattern in *P. aeruginosa*. Various plant extracts alter the expression of the *toxA* gene. For example, in a study conducted by Abbasi *et al.*, *Satureja khuzestanica* essential Oil reduced the expression of the Exotoxin A gene, resulting in reduced drug resistance in *Pseudomonas aeruginosa* [25]. In a similar result, our primary analysis revealed that the *toxA* gene expression is decreased at most concentrations of both plants' extract. The results showed that compared to the control group, the *toxA* gene was significantly ($P < 0.05$) downregulated in all isolates at all concentrations of *P. major* extracts except for C-isolate; the gene was in an overexpression pattern (Fold = 29.753, $P = 0.000$) in 250 mg/mL. Furthermore, this gene was significantly downregulated when bacteria were exposed to *Planceolata* extracts (except A and C-isolates). The gene was in an overexpression pattern at 62.5 mg/mL and 250 mg/mL of plant extract concentrations, respectively).

Furthermore, current research findings have shown that the crude extract of *P. lanceolata* and *P. major* have considerable potential in controlling *Pseudomonas aeruginosa* pathogenicity to be effective at the level of Exotoxin A expression. Gaines *et al.* claim that the combination of stagnant growth and anaerobic conditions causes *P. aeruginosa* to considerably increase *toxA* expression [34]. Exotoxin, A gene (*toxA*) expression in *P. aeruginosa*, is a complex process involving numerous regulators, including *ptxR*, which boosts *toxA* expression by 4- to 5-fold [36].

Therefore, in future studies, the effect of Plantago extract has been investigated under different conditions and on other components related to the *toxA* gene. *Plantago major* contains several active compounds such as flavonoids, polysaccharides, terpenoids, lipids, iridoid glycosides, and caffeic acid derivatives. One of the flavonoids, Luteolin, can suppress leukocyte migration and inhibit mast cell degranulation, which can be considered anti-urticaria treatment strategies. Apigenin is another flavonoid in *Plantago* species that is similar to Luteolin's cytotoxic effects [33]. On the other hand, although molecular modelling and dynamics studies predicted that Apigenin and Luteolin targets exotoxin A, Luteolin ligand with a lower dose of toxicity could be a potential drug for the treatment of *Pseudomonas aeruginosa* infections. The luteolin 7-glucoside is a potent inhibitor (IC₅₀ 40.2 mM), while apigenin 7-glucoside had no inhibitory effect on HIV-reverse transcriptase[17].

Conclusion And Future Prospective

It has been shown that crude extracts of both *P. lanceolata* and *P. major* plants have the highest capability for antibacterial activity. Generally, the outcomes of this research have shown that the tested plant extracts can control the Exotoxin A gene regulation pattern and pathogenicity of *Pseudomonas aeruginosa*, as well as Luteolin ligand with a lower dose of toxicity could be a potential drug for the treatment of *Pseudomonas aeruginosa* infections.

Declarations

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Competing Interests

"The authors have no relevant financial or non-financial interests to disclose."

Author Contributions

All authors contributed to the study's conception and design. Seyyedeh Mahdokht Maddah did supervision, statistical analysis, contributed to the manuscript's writing and editing, and performed some of the tests. Asieh Abbasi conducted Methodology and performed the experimental work performance and edited manuscript. Dhuha Saeed Ali prepared writing drafts preparation and performed Bioinformatics analysis. Mohammad Kamalinejad conducted methodology of extractions.

Data Availability

All raw data are available.

Ethics approval

Not applicable

Consent to participate

"Informed consent was obtained from all individual participants included in the study."

Consent to publish

All contributors read the manuscript and confirmed all information to submit.

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Tables

Table 1. Ligands and protein structure obtained from PubChem and RSCB Protein Data Bank and related information

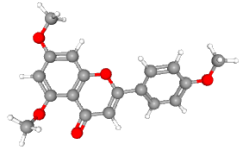
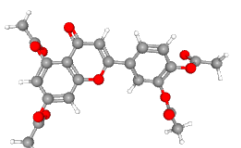
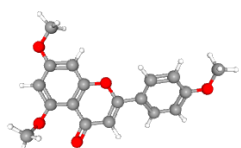
Name	PubChem/PDB ID	Molecular Formula	Synonyms	Molecular Weight	3D-Structure
Apigenin	79730	C18H16O5	Apigenin trimethyl ether, 5,7,4'-Trimethoxyflavone Trimethylapigenin	312.3	
Luteolin	135952	C23H18O10	7-acetyloxy-2-(3,4-diacetyloxyphenyl)-4-oxochromen-5-yl acetate Luteolin peracetate	454.4	
Exotoxin A	RCSB PDB/1IKQ	Data Snapshot	Pseudomonas aeruginosa Exotoxin A, wild type	66.99 kDa	
		Resolution			
		R-Value Free			
		1.62 Å	0.235	0.209	

Table 2. Antibacterial activity of the various extracts from plants on four bacteria species and Standard bacteria (*Paeroginosa* strain ATCC 9027) .The largest diameter of the inhibition zone was observed from crude (total = 1000 mg) extracts of *P. lanceollata*. The largest diameter of the inhibition zone was observed from total extracts of *P. lanceollata*, which showed inhibition zones close to that of positive control. Streptomycin (10 µg/disc) and DMSO were served as the positive and negative control, respectively.

Plants Species	Isolates	Inhibition zone diameters (mm)				
	Extracts	A	B	C	D	Standard
<i>P. major</i>	Water	14	15	15	15	13
	Petroleum ether	-	-	-	-	-
	Chloroform	-	-	-	-	-
	1000	15	15	16	16	17
	Total 500	13	10	14	14	14
	(mg) 250	-	-	-	-	-
<i>P. lanceollata</i>	125	-	-	-	-	-
	Water	19	17	15	18	15
	Petroleum ether	-	-	-	-	-
	Chloroform	-	-	-	-	-
	1000	20	20	18	17	16
	Total 500	17	18	16	16	13
Negative control	(mg) 250	15	17	15	15	12
	125	13	14	-	-	-
	Water	-	-	-	-	-
	Petroleum ether	-	-	-	-	-
Positive control	Chloroform	-	-	-	-	-
	Total	-	-	-	-	-
		21	21	21	21	21

Table 3. MBC and MIC of *P. major* and *P. lanceolata* extract materials on pseudomonas isolates

Table 4. Site-specific docking properties; ADMIT analysis and Toxicity prediction parameters.

Plants Species		MBC and MIC of antimicrobial agents (mg/ml)									
	Isolates	MIC					MBC				
	Extracts	A	B	C	D	STD	A	B	C	D	STD
	Water	125	125	125	125	125	125	250	125	125	125
<i>Plantago major</i>	Total	62.5	62.5	62.5	62.5	62.5	62.5	250	62.5	62.5	62.5
<i>Plantago lanceollata</i>											
	Water	15.62	15.62	15.62	15.62	15.62	15.62	15.62	31.25	15.62	15.62
	Total	7.81	15.62	7.81	7.81	15.62	125	62.5	62.5	62.5	125

Compounds/ Ligand information										cIRMSD	refRl
Predicted LD50	Predicted Toxicity Class	Average similarity	Prediction accuracy								
Ligand's properties		Hydrogen bond acceptors	Hydrogen bond donors	Number of atoms	Number of bonds	Number of routable bonds	Molecular refractivity	Topological Polar Surface Area	Octanol/water partition coefficient(logP)	0.03	66.8
Apigenin (CID_79730)	312.32	20	0	39	41	4	87.4	57.9	3.49		
Luteolin (CID_135952)	454.38	27	0	51	53	9	113.92	135.41	3.16	0	67.9

Figures

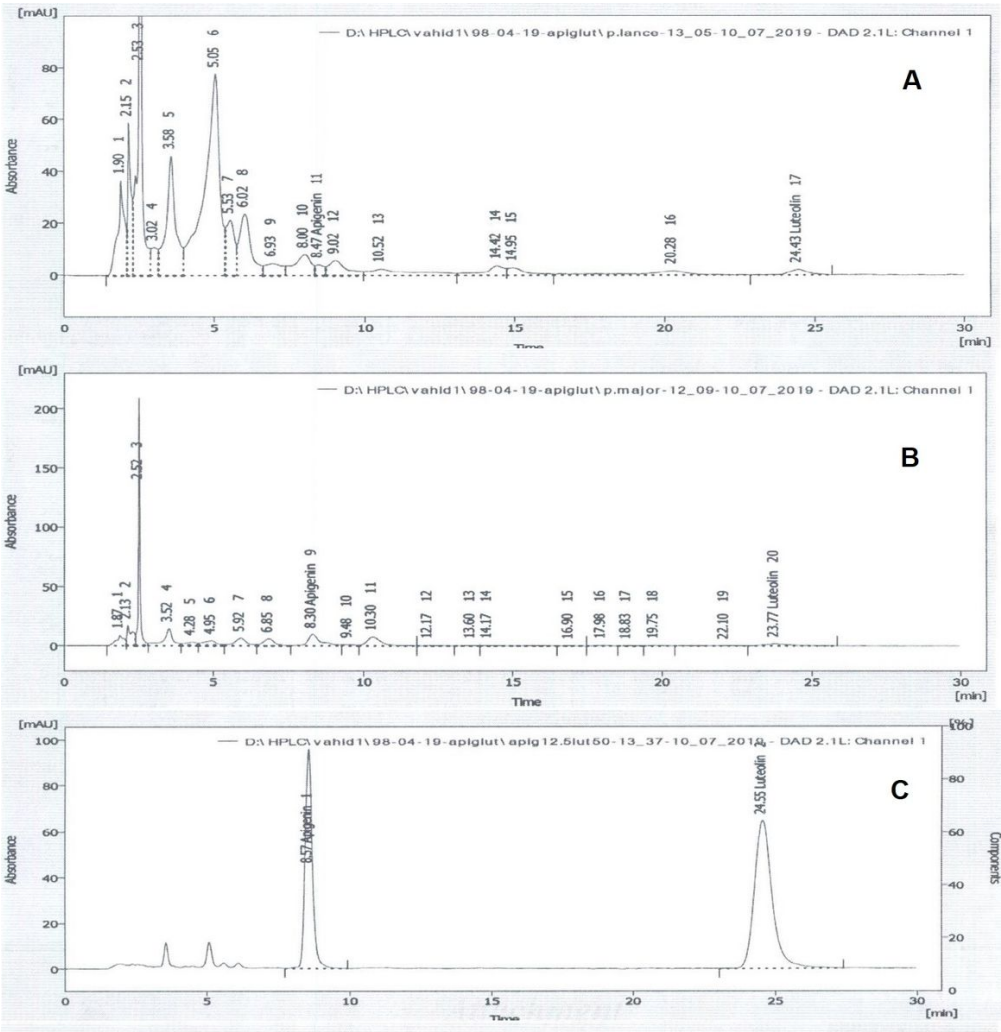


Figure 1

Isolated Apigenin and Luteolin from *Plantago lanceolata* (A) and *Plantago major* (B) using the isocratic HPLC method. (C) served as standard.

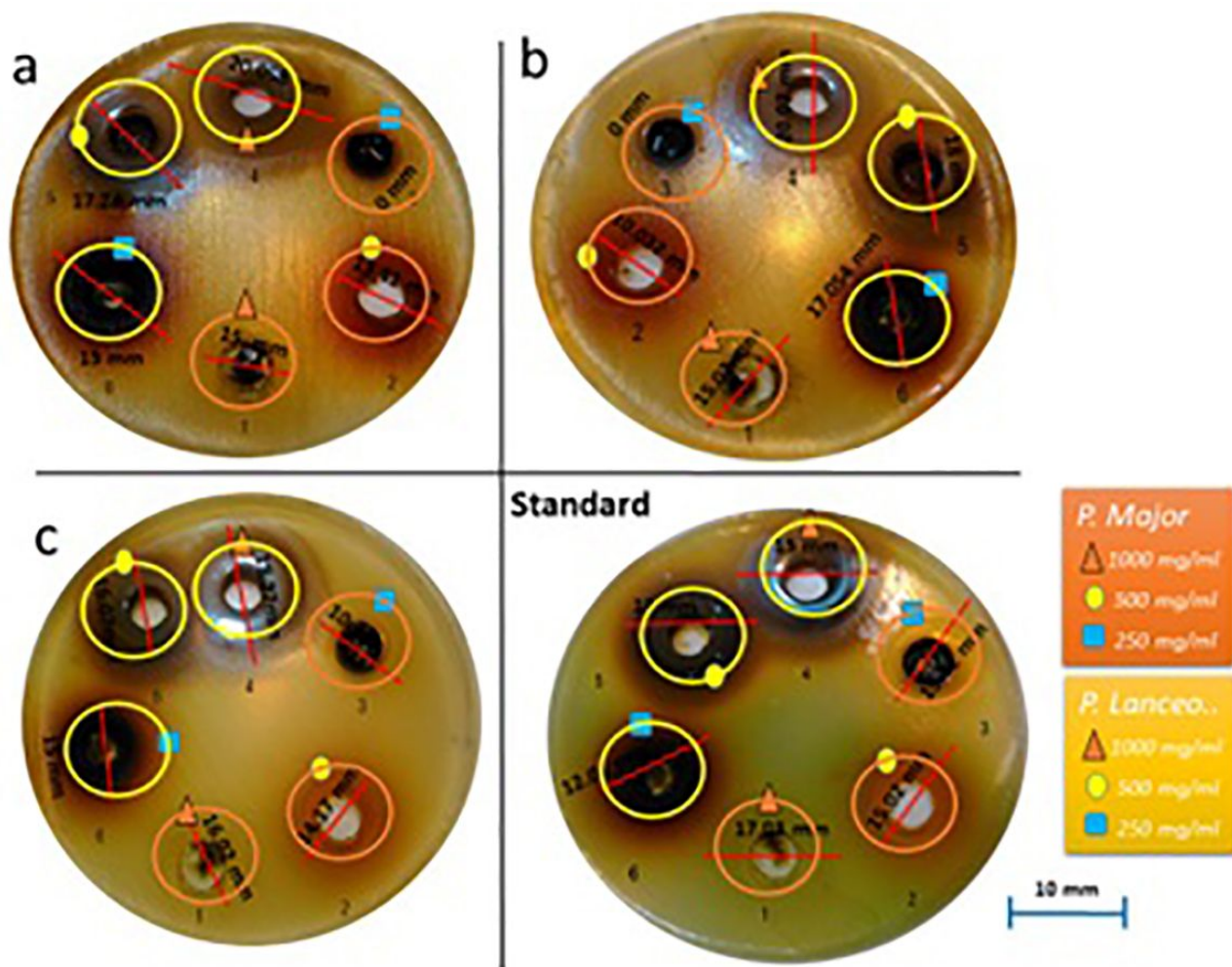


Figure 2

A schematic presentation of antibacterial susceptibility test results of *P. major* and *P. lanceolata* extract materials. The inhibition zones were measured using MIP software. Orange and yellow circles indicated *P. major* and *P. lanceolata* effects. The concentration value in each inhibition zone is presented in color symbols. In species a and b, the concentration of 250 mg/mL of *P. major* did not show any antibacterial effects.

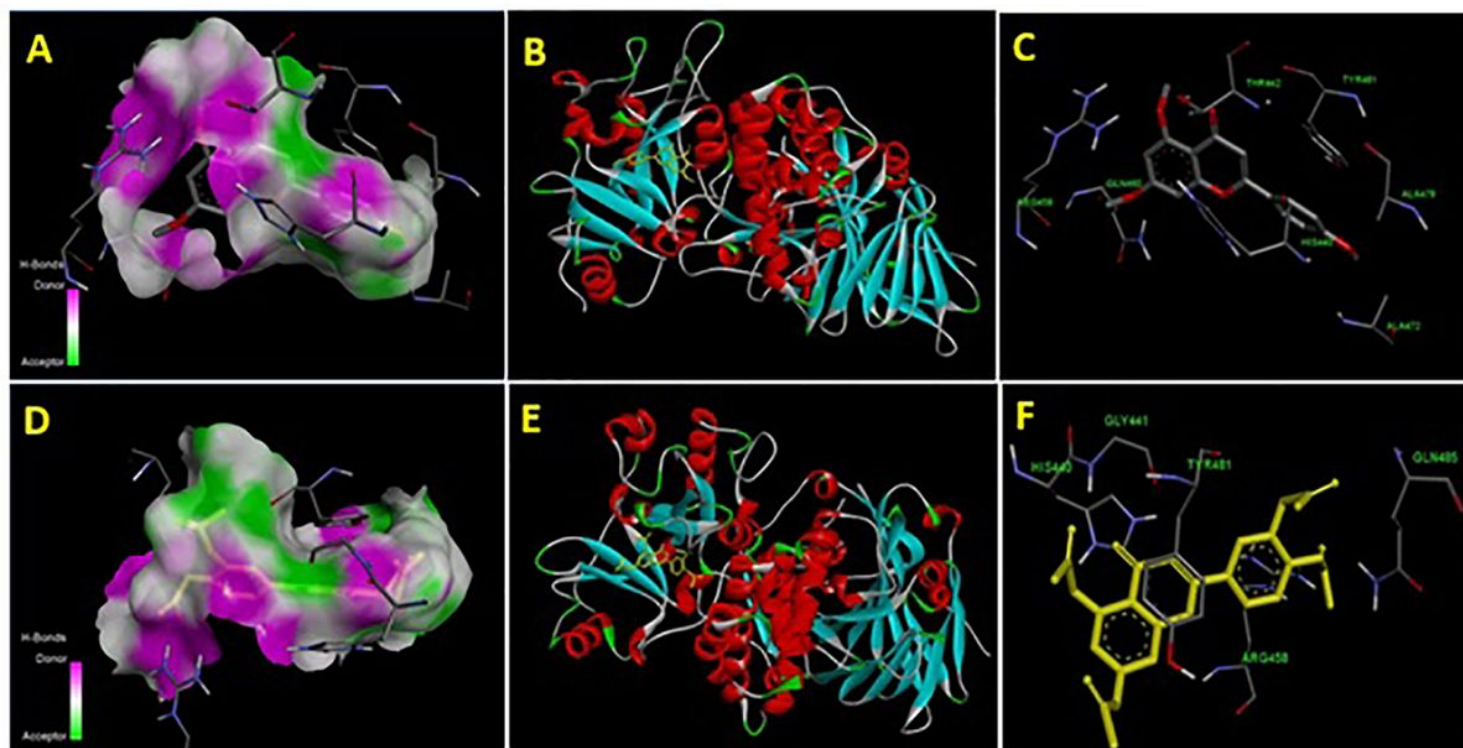


Figure 3

A schematic presentation of molecular modeling inhibition of ligands and Exotoxin-A protein and amino acids residue interactions. Donor and acceptor hydrogen bands presented in ligands (Apigenein (A) and Luteolin (D)). The best pose interaction pattern between Apigenein-EXO-A (B) and Luteolin-EXO-A(E). Residue interactions in Apigenin (C) and Luteolin (F) ligands. Apigenin at ARG458, THR442, GLN460, HIS440, TYR481, ALA478, ALA472 residues, and Luteolin at GLY441, GLN485, ARG458, HIS440, TYR481 residues interact with Exotoxin-A protein

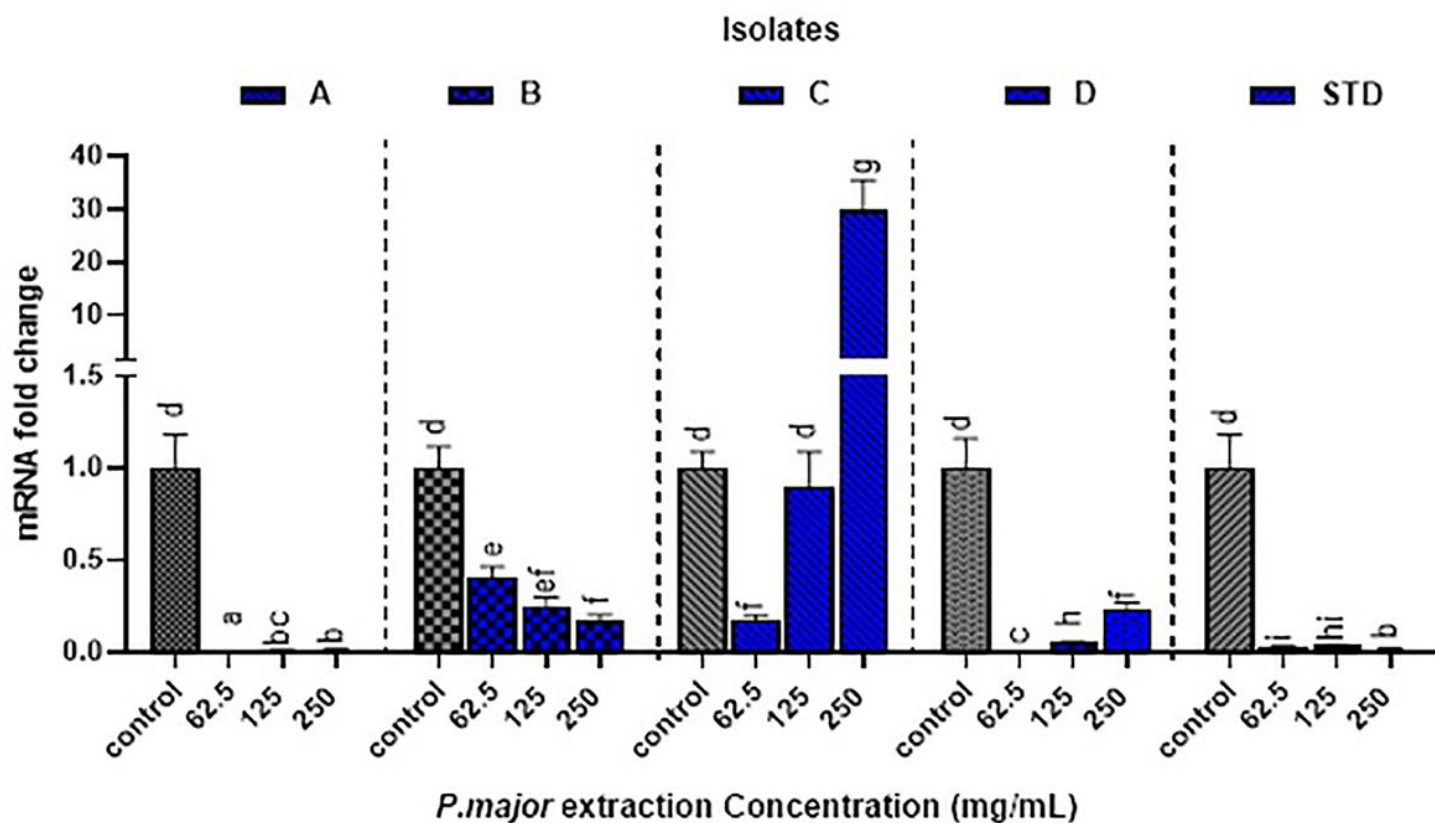


Figure 4

Schematic presentation of the *tox*A gene expression value after exposure of *Pseudomonas aeruginosa* with *P. major* in different concentrations of extracts materials. The results show that compared to the control group, the *tox*A gene significantly ($P < 0.05$) downregulated in all isolates at all concentrations of *P. major* extracts exception of C-isolate that the gene was in an overexpression pattern (Fold =29.75376, $P =0.000$) in 250 mg/mL.

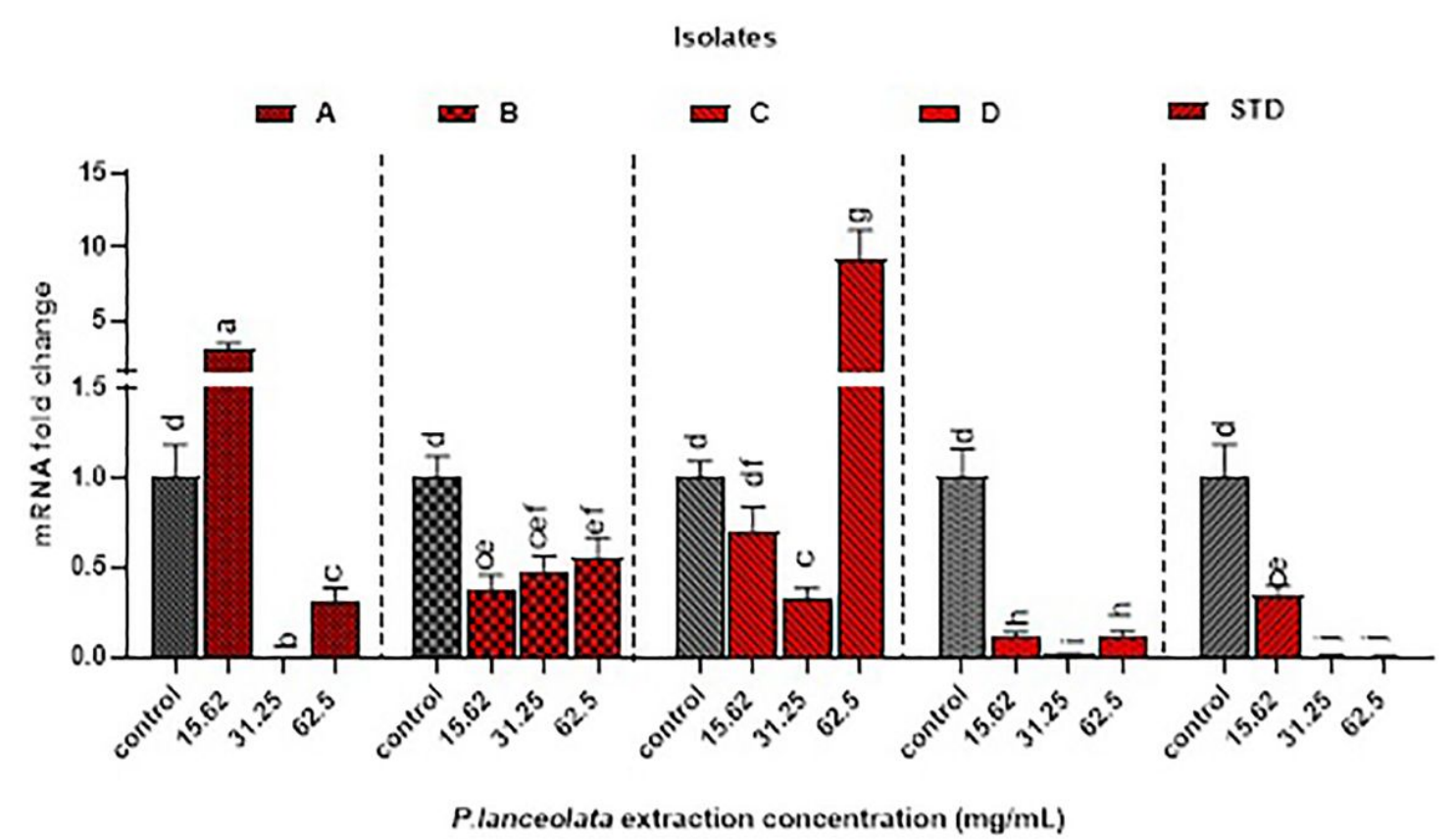


Figure 5

Schematic presentation of the *tox*A gene expression value after exposure of *Pseudomonas aeruginosa* with *P. lanceolata* in different concentrations of extracts materials. The results show that compared to the control group, the *tox*A gene significantly ($P < 0.05$) downregulated in all isolates at all concentrations of *P. lanceolata* extracts exception of A and C-isolates that the gene was in an overexpression pattern at 62.5 mg/mL and 250 mg/mL of plant extract concentrations respectively.

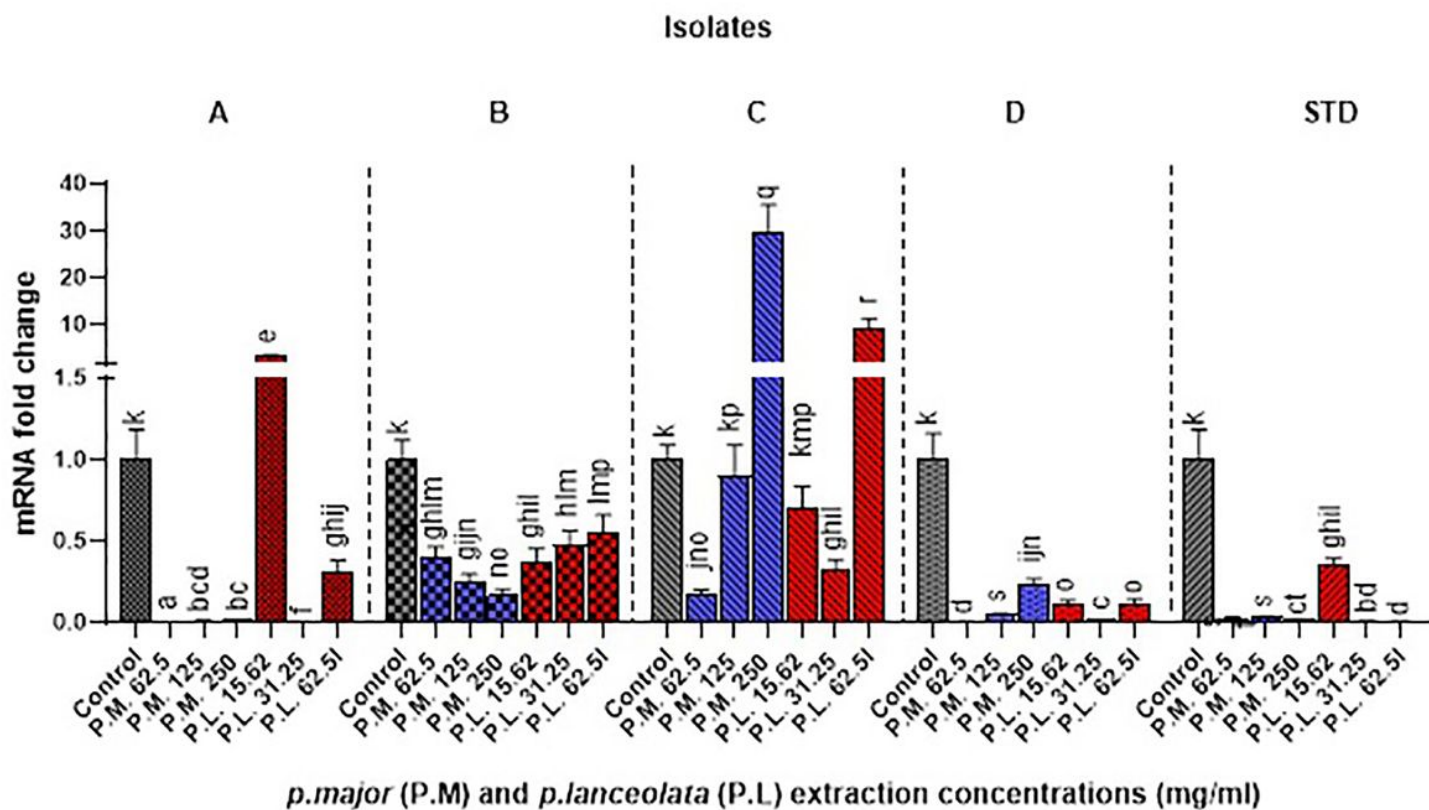


Figure 6

A schematic of comparison diagram of the *toxA* gene expression in different strains in the most concentration of extracted materials, compared to the control group, Exotoxin A gene significantly downregulated ($P < 0.05$). Gene expression of control samples with gray color, samples treated with different concentrations of *P. major* (P.M) total extract with blue color, and samples treated with varying concentrations of *P. lanceolata* (P.L) total extract with red color. The presence of similar letters on the charts show that the observed difference is not significant. The *toxA* gene expression is decreased at most concentrations. In strain A, gene expression increased at a 15.62 mg/mL concentration of *P. lanceolata*. Extract. In strain C, a significant increase in the *toxA* gene expression was observed under the influence of the highest concentration of extracts of the two plants.