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Broad spectrum anti-quorum sensing activity of traditional medicinal plant: *Piper betel*

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Abstract
Piper betel Linn, a traditional herb is well documented for its medicinal importance in the ancient book of Ayurveda (3000 BC). The leaf part of the plant is full of bioactive phenols and terpenes and consumption of its leaf show a varied range of pharmacological activities such as antimicrobial, stimulant gastroprotection and anti-parasitic effect. However, *P. betel* antimicrobial property attribution with anti-quorum sensing (QS) activity has not been explored much. In the present study, first time broad spectrum anti-QS activity of 50% hydroalchoholic extract of *P. betel* fresh leaf was explored using *Chromobacterium violaceum* (CV) mutant strains (ATCC 12472, MCC 3299 and MCC 2216), *P. aeruginosa* (PA01) and *C. albicans*. In experimental results, Pb-Extract showed strong Quorum quenching behavior against CV 12472 at concentration range 100-300 µg without affecting bacterial growth. Also, Pb-Extract treatment against PA01, showed a marked reduction in virulence factors like pyocyanin, protease, rhamnolipid and alginate. A significant reduction in biofilms production was also reported against PA01 and *C. albicans* at all range of Pb-Extract dose. Further, *luxI*/*luxR* effect data revealed that quorum quenching property of Pb-Extract is associated with inhibition of autoinducer (AHL) production and AHL binding receptor synthesis both. Since, QS mechanism modulates bacterial infection pathogenicity via expression of diversified virulence factors. Therefore, potential anti-QS agents are considered as a better approach for broad spectrum antibacterial drugs. These drugs are free from any side effects for creating drug resistance in pathogens via selective pressure. The present study proposed *P. betel* as an edible herbal source for a potent anti-QS agent with broad range of QS quenching properties.

Keywords: *P. betel*, quorum quenching, virulence factors, *luxi*/*luxr* effect

Introduction

Piper betel Linn. (Family Piperaceae) is an evergreen shade loving, climbing shrub. Its leaves are widely used as masticators for mouth freshener and sweeten the breath. The leaves have been used for traditional medicinal purposes in Asiatic region, particularly in south-East Asia. In India *P. betel* leaves fold with other ingredients like seeds of (Areca catechu L), Limestone, sweetening jelly is used for mastication, popularly known as betel quid (or Paan). Medicinally *P. betel* leaves consumption is said to be showed activities such as antibacterial, antifungal, anthelmintic, laxative, gentle digestive stimulant, antifilarial, antiprotozoal activity, antiallergic effects, hepatoprotective effects, antiulcer effects (gastroprotection), cardioprotective effects, improve immune response, antimutagenic effects and radioprotective effects[1-11]. Also, *P. betel* leaves formulations with other plants are used for various remedies including aphrodisiac activity as texted in the ancient book Ayurveda [12]. *P. betel* contains many bioactive phenols and terpenoids. Its secondary metabolites and essential oil contains key phytochemicals such as chavicol, chavibetol, eugenol, estragole, hydroxycatechol, caryophyllene, cadinene, catechol, cepharadione A, γ-lactone, isoeugenol, β-pinene, β-sitosterol, ursolic acid, monoterpenes (terpinene and p-cymene) and monoterpenoids (carvacrol and eucalyptol) [13].

It is stated in an ancient book of Vedic period (3000 BC) that extracts of *P. betel* plants from different parts showed effective antimicrobial activity against various pathogenic microorganisms [5]. Also, people of India use betel quid to cure candidal infection in oral cavity. Moreover, recent studies suggested that the antibacterial activity of *P. betel* is attributed to its Quorum sensing (QS) effect [14]. QS, a key communication mechanism that determines the pathogenicity of bacterial infection by activating virulence factors production. The QS phenomenon is triggered through a population density dependent manner, caused production of some signaling molecules known as autoinducers (AIs). In gram-negative bacteria *luxI* gene system encodes the production of autoinducer AHL (*N*-acylated homoserine lactones) which on a certain concentration level activates *luxR* gene system that synthesized AHL receptors. This AHL-receptor complex binds with QS regulating promoters and activates the expression of QS regulated virulence genes and production of virulence factors such as extracellular Proteases, toxins, motility, and biofilms formation. Virulence factors plays important role in bacteria attachment, surpass host immune defense and antibiotic resistant, collectively increase pathogenicity [15, 18]. Therefore a potential anti-QS agent could be served as a new type of antibacterial agent that curb bacterial pathogenicity with a minimum chance of getting molecular modulation for antibiotic resistant.

In the present study anti-QS potential of Pb-Extract was explored using the biomonitor strain of *Chromobacterium violaceum* (CV) 12472. In addition, antibiofilm effect of *P. betel* leaf extract was analyzed against *Pseudomonas*

aeruginosa (PA01) and *Candida albicans*. The further anti-QS activity of *P. betel* extract was checked via QS regulated gene system *luxI*/*luxR* system, to evaluate whether its activity relies on inhibition of AHL production or production of AHL receptor using *C. violaceum* mutant strains (MCC 3299 and 2216).

Materials & Methods

Collection of *P. betel* leaves

Piper betel fresh leaves were purchased from the local shop of Lucknow district of Uttar Pradesh India.

Preparation of *P. betel* leaf extract (Pb-Extract)

P. betel leaves were washed with distilled water (DW) and allow drying at room temperature to remove wetness. Then 50 g of leaves were chopped by sterilized blade cutter and crushed in a mortar pestle. The leaf paste was mixed with 100 ml of 50% hydroalcoholic solvent and kept on a rotator shaker at 50 rpm for 24 hrs for a complete extract of metabolites. Then the mixture was centrifuged at 5000 rpm for 5 min and filtered through whatman filter paper (0.45µm pore size). The filtrate was left to dry at RT and dried extract stored at 4 ºC for further use.

Microbial strains and culture maintenance

The biomonitor strains used in experiments are:

a. *C. violaceum* ATCC 12472 a wild type strain produces purple color pigment (violacein) in response to QS mediated autoinducer (C6 AHL).
b. *C. violaceum* MCC 3299 and *C. violaceum* MCC 2216, NCCS Pune India. The former strain MCC 3299 is a non-pigmenting mutant strain but modified to overproduce N-hexanoyl-L-homoserine lactone (C6-HSL). The latter strain MCC 2216 is mini Tn5 mutant of *Chromobacterium violaceum* 31532 and unable to synthesized C6-HSL its own, but produced violacein when stimulated by exogenously provided C6-HSl.

The *C. violaceum* strains ATCC 12472, MCC 3299, MCC 2216 and *P. aeruginosa* (PA01) culture stock were maintained in Nutrient agar slant at 4 ºC. Whereas *C. albicans* culture stock was stored in sabouraud dextrose agar (SDA) slants. The cultures were revived from their stock preparation in Luria-Bertani agar (bacterial strain) at 37 ºC and SDA (*C. albicans*) at 30 ºC for 24 hours. Then single pure colony of all strains was analyzed under a microscope and cultured in LB broth (bacteria) and SD broth (*C. albicans*). All the experiments were performed using standard cell suspension (SCS) of respective strain, prepared by adjusting cell population to 0.5 at OD_{600} in 0.85% saline.

Determination of anti-QS activity

The anti-QS activity of 50% hydroalcoholic Pb-Extract was examined by using disk-diffusion overlay method using biomonitor strain CV 12472 [19]. Briefly, 50 µl of 0.3% LB agar was prepared and into this SCS of an overnight grown broth culture of CV 12472 was mixed when agar was in a molten state (30-35 ºC) and poured this mixture over pre-cooled LB agar plated (1.5% agar) so that the mixture was spread evenly, forming an upper layer. On solidification of agar plates, 6 mm sterile paper disks were placed on it and various concentration of Pb-Extract (100, 200, and 300 µg dissolved in 5 % DMSO) was loaded (15 µl). The agar plates were incubated at 37 ºC for 24 hours and anti-QS activity was determined by observing a clear halo zone formed around the treated disk due to inhibition of violacein production.

Quantification of Violacein production

C. violaceum 12472 produces QS mediated violet pigment violacein. Therefore inhibition of violacein production indicated QS inhibition. We determined violacein production in Pb-Extract treated culture by the method of Singh *et al*. SCS of overnight grown *C. violaceum* 12472 culture were inoculated in 50 ml LB broth and treated with various concentrations of Pb-Extract followed by incubation of 24 hours at 37 ºC. Violacein production was quantified by extracting violacein through cell lysis with 10% SDS treatment for 10 minutes. Then water-saturated n-butanol was added, mixed vigorously and centrifuged to separates cell debris and violacein dissolved in organic layer. Extracted violacein absorbance was taken at wavelength 585 nm for quantification [20].

Biofilm inhibition assay

The Anti-biofilms effect of Pb-Extract was analyzed in 6 well plates using *P.aeruginosa* (PA01). An autoclaved glass slides was placed in wells and 2 ml freshly prepared sterile LB broth was added. To this broth media over-night grown PA01 culture SCS was used to inoculate and each well was treated with different concentrations of Pb-Extract (100, 200 and 300 µg). Whereas control well was left untreated. The culture plates were allowed to incubate at 37 ºC for 24 hours statically. After biofilms formation excess media in the well was discarded carefully without disrupting biofilms and washed twice with phosphate buffer saline (PBS) 7.4 pH and fixed with 70% isopropanol. Biofilms adhere to glass slides were stained with 10% v/v crystal violet stain. Similarly, SD broth media (100 µl) was placed on cavity slides and overnight grown *C. albicans* culture SCS was used to inoculate media and treated with a respective concentration of Pb-Extracts. After 24 hours incubation at 30 ºC media was discarded and formed biofilms stained with 20% v/v crystal violet stain [20]. The stained biofilms were analyzed under bright field microscopy (Leica DM2000 & DM2000 LED). For quantification of biofilms inhibition, crystal violet stained biofilms (adhere on glass slides) were resuspended in 0.5 ml ethanol (95%) for 5 min and absorbance was taken at 650 nm.

Effect of *P. betel* extracts on QS regulated virulence production in *P aeruginosa*

P. aeruginosa produces various virulence factors that are regulated through QS-mechanism such as pyocyanin, proteases, rhamnolipid and alginate. The virulence factors facilitate overall pathogenicity and antibiotic drug resistance ability during infection. For determination of Pb-Extracts effect on virulence production, overnight grown *P. aeruginosa* culture inoculated in sterile Luria broth in 250 ml flask, supplemented with various concentrations of Pb-Extracts. Whereas the control culture flask was left untreated. The flask culture was incubated on a rotator shaker at 100 rpm, 37 ºC for 24 hours. Then culture was centrifuged at 5000 rpm for 10 minutes and filtered through a syringe filter (0.45µm) to remove any bacterial cells. Pyocyanin production was estimated by mixing 4 ml cell-free supernatant (CFS) with 2 ml of chloroform followed by vigorous shaking. From this 1 ml of organic layer was

mixed with 0.3 ml of 0.2 N HCL. A light pink color was developing in the upper layer of the solution. Pyocyanin production was determined by taking absorbance of the upper layer at OD_{520} [21]. For protease activity 100 µl supernatant of treated culture was mixed with 900 µl of 0.5% azocasein substrate prepared in solution (50 mM Tris buffer and 2 mM $CaCl_2$) and the reaction mixture left for incubation for 1 hour at 30 ºC. 10% TCA (100 µl) was used to stop the reaction and mixture was centrifuged at 8000 rpm for 10 minutes. The absorbance of this supernatant was taken at 440 nm [22]. Rhamnolipid production was determined by the orcinol method, described earlier [23, 24]. Alginate production was estimated by precipitating culture supernatant through isopropanol. Then the precipitate was centrifuged and the dried pellet was resuspended in DW and 0.2% carbazole reagent dissolved in 10 mM boric sulfuric acid. After 15 min the absorbance of the reaction mixture was measured at 500 nm [25].

Determination of *luxI*/*luxR* inhibition effect

We determined whether the anti-QS activity effect of Pb-Extract was associated with inhibition of *N*-acyl homoserine lactone response (AHL) synthesis (*luxI* effect), or due to effect on AHL binding receptors (*luxR* effect). Briefly, bacteria strains *C. violaceum* MCC 2216 and MCC 3299 were streak on Luria agar plates in the form of two semi concentric circles with 8-10 mm apart such that in one half of the plate strain MCC 2216 form inner circle and another half MCC 3299 form inner circle. A 6 mm sterile paper disk was placed inside each inner circle of bacterial streaking and 25 µl (300 µg) of Pb-Extract was placed on the disk. Then plates were allowed to incubate at 37 ºC for 24 hours. The *luxI*/*luxR* inhibition effect was determined through violacein pigment production in MCC 2216 strain. A weak signal of violacein pigmentation in MCC 2216 when it forms the outer circle indicates that *luxI* was the target whereas the lowered signal of violacein production in MCC 2216 when it forms the inner circle indicates *luxR* was the target [26, 27].

Results

Despite of diversified human health benefits effects *P. betel* pharmacological properties have not been explored much. In present study, hydroalcoholic extract of *P. betel* fresh leaves was evaluated for its broad spectrum quorum quenching and antivirulence activites.

Screening for anti-QS activity of Pb-Extracts and quantification of violacein production

Anti-QS activity was checked by using biomonitor strain *C. violaceum* 12472. The bacteria produce violet color pigment violacein regulated by QS. Since Inhibition of violacein does not exert any growth restriction, therefore considered as a marker for QS inhibition. Anti-QS activity was analyzed by observing a clear halo zone formed around the sample loaded disk due to violacein inhibition. Results showed strong activity of Pb-Extract in dose-dependent manner at all concentrations (100-300 µg) figure 1(A). However, no significant growth inhibition was found at all ranges of Pb-Extract concentration, which indicated that the activity of the test sample was associated with only QS-inhibition. Further violacein production in treated culture was quantified by taking absorbance of extracted violacein at wavelength 585 nm figure 2(B & C)). Results indicated a significant decrease in pigment production in treated culture with increased inhibition at each increment in dose. We observed maximum activity at 300 µg concentration with 71 % (after 24 hours) and 75% (after 48 hours). Interestingly, we found that *C. violaceum* 12472 cells density was continuously increased within broth culture but newly generated cell also not able to produce violacein. This suggested that Pb-Extract activity must be linked with inhibition of QS-regulated genes at initial levels.

Inhibition of biofilms formation

Biofilm formation is QS-mediated mechanisms that facilitate pathogenic microbes to adhere surfaces, protect against the host immune environment and plays a key role in antibiotic resistance. *P. aeruginosa* and *C. albicans* both is excellent biofilms producers. Therefore we experimented on to determined anti-biofilm property of Pb-Extract in their static culture. Bright filed microscopic image results showed that in control culture, biofilms formed on glass coverslips is dense, thick and continuous with abundance of exopolysaccharides in both strains, figure 1(C). Whereas, in extract treated culture we observed discontinuous, distorted, thin biofilms formation at all range of concentrations (100-300 µg). Maximum destruction of biofilms was observed at concentration 300 µg. Further quantification of biofilms production results showed 60% (100 µg), 77% (200 µg) and 92 % (300 µg) inhibitions in *P. aeruginosa*. Similarly in *C. albicans* 44.5 % (100 µg), 69% (200 µg) and 87% (300 µg) inhibition was observed, figure 1(B). Similar activity with significant antibiofilm property was reported in previous study where hydroalcoholic extract of *P. betel* dried leaves showed 75.35% reduction of biofilms production against *P. aeruginosa* at 200 µg [14].

Effect of Pb-Extract on QS regulated virulence production in *P. aeruginosa*

P. aeruginosa produced virulence factors in response to QS mediated expression of various virulence genes. A potential anti-QS agent, downregulate the production of virulence factors and make pathogenic bacteria more susceptible to antimicrobial drugs. To assess Pb-Extract activity against pyocyanin production supernatant of 24 hours old treated and untreated (control) broth culture subjected to a biochemical test for quantitative analysis. As shown in figure 2(A), at 300 µg Pb-Extract significantly suppress the production of pyocyanin by 67%. However, activity was also reported at 100 µg (31%) and 200 µg (42%) inhibition compared to untreated control. For protease activity, azo-casein substrate degradation in the treated and untreated culture supernatant was determined by spectrophotometer absorbance at OD_{440}. Results indicated that protease activity was decreased by 34% (200 µg) and 40.5% (300 µg). Similarly, rhamnolipid and alginate production was also significantly decreased when exposed to Pb-Extract. We reported 60% and 57% drop in rhamnolipid and alginate production, compared to the control culture supernatant, figure 2 (A).

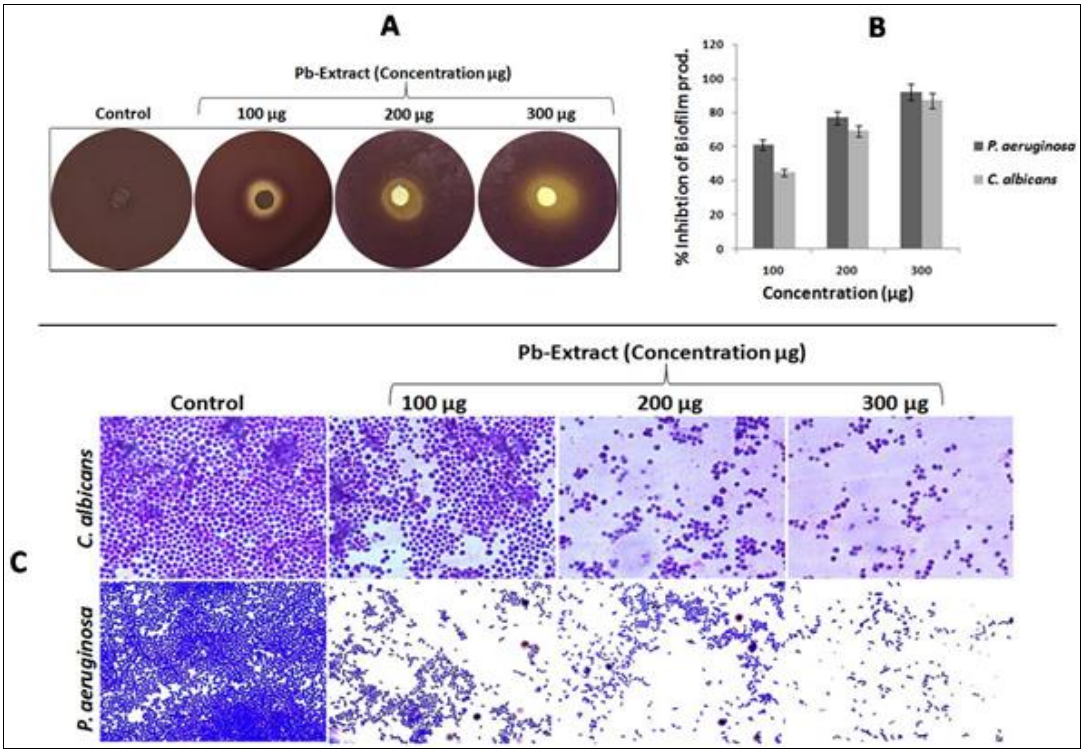

Fig 1: (A) Anti-QS activity of Pb-Extract on biomonitor strain CV 12472. (B) Percentage inhibition of biofilm production determined through crystal violet test. Error bar indicates SD of 3 independent experiments. (C) bright field microscopic images of Pb-Extract treated biofilms formed on glass coverslip (i) *C. albicans* and (ii) *P.aeruginosa*

luxI/*luxR* inhibition effect

To determine whether the effect of Pb-Extract as anti-QS agent is associated with intervention in the production of autoinducer HSL (*luxI* inhibition) or its transcriptional response promoter (*luxR* effect), two biomonitor mutant strains were used, figure 2 (D). Results indicated that CV 2216 does not produce violacein (when in the inner circle) in response to HSL produced by CV 3299, suggested that extract interfere with the receptor binding of HSL (autoinducer) to transcription response promoter and inhibited expression of QS genes (i.e. luxR effect). Subsequently, CV 2216 does not produce pigment when present in the outer circle indicated that extract inhibited the production of HSL in CV 3299, representing a significant *luxI* effect.

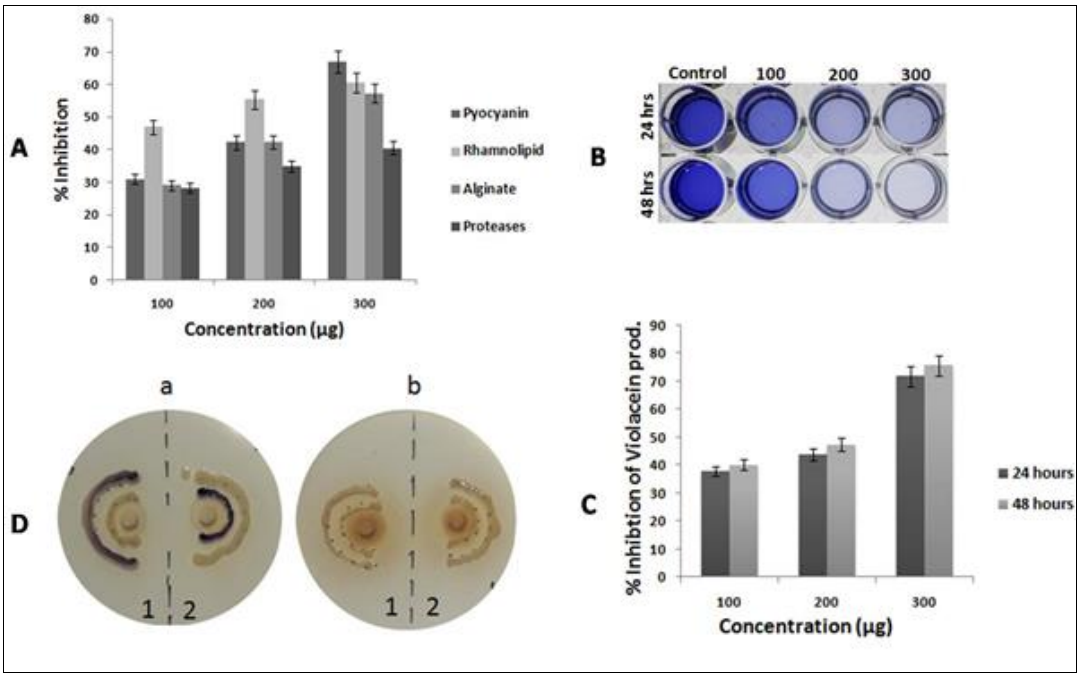

Fig 2: (A) Effect of Pb-Extracts on QS-regulated virulence production in *P. aeruginosa* (B) Violacein extracted from Pb-extract treated cultures. (C) Percentage inhibition of violacein produced in Pb-Extract treated culture. (D) Showed *luxI*/*luxR* effect. (1) Represent CV 2216 strain in the outer circle and CV 3299 in the inner circle and (2) vice-versa. (a) Indicated untreated control where CV 2216 produced violacein pigment in response to AHL produced by CV 3329. (b) Pb-Extract affects both AHL production and receptor binding of AHL (i.e autoinducer activity). Error bars indicated SD of three Experiments.

Several natural compounds and plant extracts have been reported for anti-QS activity [28, 30]. They show anti-QS activity either by suppressing autoinducer (AHL) activity due to camouflage molecular structure or by interfering with *luxR*/*lasR* AHL receptors [31, 32]. The present study first time demonstrated through *luxI*/*luxR* effect of Pb-Extract, that its activity is a combined effect of AHL activity and interference of AHL synthesis. Phytochemically *P. betel* leaves contains many biological active phenols, terpenes and essential oils Therefore anti-QS property of *P. betel* extract is also the cumulative effect of its bioactive metabolites. A similar anti-QS effect was observed in crude ethanolic extract of *P. betel* leaf with significant inhibition of *P. aeruginosa* virulence factors (pyocyanin, motility and biofilms production) at 200 µg concentration [14]. In QS phenomenon microorganism shows the formation of biofilms, which is crucial for pathogenic microbe for survival in an unfavorable host environment, antibiotic resistant and surface adherence. *P. aeruginosa* can able to form biofilms on metal surfaces of medical utensils, so as *C. albicans* [33-35]. Pb-Extract not only inhibits QS but also suppresses the production of virulence factors. In the anti-biofilm experiment, results showed significant inhibition of biofilms formation in both *P. aeruginosa* and *C. albicans*. Inhibition of biofilms production directly makes microbe susceptible to antibacterial drug. Therefore Pb-extract demonstrated its potential as strong anti-QS agents and could be used synergistically with conventional anti-microbial drugs against multidrug resistant pathogens. However, the exact mechanism for Pb-extract activity against *C. albicans* was not evaluated. Since masticating Betel quid during *Candida* infection in the mouth is common practice in India, therefore one hypothesis suggests that this activity could be indirectly related with Pb-extract gastrointestinal effect which favors flourishing of intestinal micro-flora and secondary metabolites produced by these microbes (probiotics) is well known for anti-candidal activity [36]. The extracellular virulence factor determines the successful establishment of bacterial infection [37, 38]. Results showed a significant drop in other virulence factors such as pyocyanin production, the virulence factor affects gene expression, metabolism and innate immune response in the host [39, 40]. Rhamnolipd and alginate provide an extracellular matrix for biofilms formation. We reported decreased production of these rhmanolipid and alginate production in Pb-extract treated culture by 60% and 57%. *P aeruginosa* produces type IV protease and alkaline protease controlled by QS genes. These exoenzymes degrade immunoglobulins, damage tissue, host complement proteins and host fibronectin, therefore promoting promulgation of bacteria over host infection [41, 42]. Protease activity in Pb-Extract treated culture was found to be reduced by 40.5 % at 300 µg. In our study, we found Pb-Extract as a potential quorum quenching agent that significantly suppresses the QS mechanisms by influencing both production and binding of auto-inducer (AHL). The study validates the traditional importance of *P. betel* leaves mastication against microbial infection.

Conclusion

The emergence of multidrug resistance among most human pathogens requires urgent review on the reliability of presently available conventional antimicrobial drugs. The instant solution of this problem is to incorporate potential anti-QS agents with antimicrobial drugs. Anti-QS agents target bacterial QS-regulated virulence factors that play a key role in the severity of pathogens infection and make them susceptible to antimicrobial drugs at a much lower dose. Subsequently, lower the process of drug resistant adaptation in pathogens. Pb-Extract demonstrated significant anti-QS activity against CV, PA01 and *C. albicans*. It curb the expression of virulence genes and hence the production of virulence factors in PA01. The data evaluated from this study presented *P. betel* as a potent anti-QS agent. However, more advanced research needed to be performed to characterized and purify bioactive molecules, their mode of action and toxicity. So that *P. betel* makes available for human welfare with scientifically proved data.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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