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Anti QS (quorum sensing) and DPPH scavenging activity of *Nasturtium offficinale* R.Br. and *Amaranthus spinosus* L.

Somali Chattopadhyay, Shohini Chakraborty and Sukanta Majumdar

Microbiology and Microbial Biotechnology Laboratory, Department of Botany, University of Gour Banga, Malda-732103 (India)

Abstract

In present study we evaluated the antioxidant activity and anti quorum sensing activity of ethanolic extracts of *Nasturtium officinale* R.Br (Brassicaceae) and *Amaranthus spinosus* L. (Amaranthaceae). The experiments conducted on the basis of the QS-I (quorum sensing inhibition) activity of plant extract against the MTCC2656 *Chromobacterium violaceum.* Extractive value of ethanolic plant extracts were 85.0% in case of *A. spinosus* and 18.5 % in case of *N. officinale.* The anti-biofilm activity of plant sample was done by the three different ways such as agar well diffusion method, flask incubation assay and bioassay for quorum sensing inhibition by TLC. In flask incubation assay, inhibition percentage (%) of violacein production by *A. Spinosus* and *N. officinale* were 87% and 82% respectively. In the thin layer directed (TLC) bio autography, zone of inhibition determined by the help of R_f values that showed two unique spots likes 0.49 and 0.51 which were responsible for the anti QS activity in *N. officinale, A. spinosus* respectively. On the basis of phytochemical studies, the result revealed that both the samples consists of carbohydrate, protein, glycosides but in *A. spinosus* phenol was absent, where as flavonoids and tannin were present. Similarly, in *N. officinale* phenol was present but flavonoids and tannin were absent. Some nutritional factors present in both plant extract such as *A. spinosus* consist of 348.27 µg/ml of total protein, 37.6 µg/ml of total soluble sugar and 63.1 µg/ml of total reducing sugar as well as *N. officinale* consist of 536.19 µg/ml of total protein, 83 µg/ml of total soluble sugar and 19.7 µg/ml of total reducing sugar. Antioxidant activity of plant sample was checked by DPPH free radical scavenging activity at 517 nm. A dose dependent DPPH scavenging activity was observed in case of both the plants. TLC showed that coumarin and saponins were present in both the sample, but the presence of tannin and flavonoid was recorded only in *A. spinosus.*

Most medical applications of plants focused on their antimicrobial and antioxidant effects with low attention towards anti-pathogenic effects[34]. Recently, research efforts are focused on controlling bacterial infection through developing anti-pathogenic

agents which manage bacterial diseases by inhibiting bacterial communication process called bacterial quorum sensing (QS). Quorum communication system regulates the release of bacterial virulence factors such as protease, elastase, pyocyanin, alginate, biofilm formation, bacterial motility, and toxins production[8,12,40]. Plants are considered as a rich natural resource of quorum quenching agents[3,16,19]. Now a days, there is growing interest in the plant based medicine because several bioactive constituents have been isolated and studied for pharmacological activities. During the last two decades pharmaceutical industry has made a massive investment in pharmacological and chemical researchers all over the world in an effort to discover the much more potent drug, rather a few new drugs. Furthermore, the use of herbal medicine for the treatment of diseases and infections is as old as mankind. The World Health Organization supports the use of traditional medicine provided they are proven to be efficient and safe[38]. In developing countries, a huge number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have no alternative for primary health care. Therefore, need to look inwards to search some herbal medicinal plants with the aim of validating ethno medicinal use and subsequently the isolation and characterization of the active principle in it. Bacteria communicate through the production of diffusible signal molecules termed auto inducers. The control of gene expression by auto inducers is cell-density-dependent; this phenomenon has been called quorum sensing. As quorum sensing controls virulence, it has been considered an attractive target for the development of new therapeutic strategies. Gram-positive and Gram-negative bacteria use different types of QS systems gram-positive bacteria use peptides, called auto-inducing peptides (AIPs), as signalling molecules[36]. When the concentration of AIs is sufficiently high, which occurs at HCD, they bind cytoplasmic receptors that regulate expression of the genes in the QS region[10,13,14,18,20,22,23,27,30]. The final processed AIPs range in size from 5 to 17 amino acids, can post translational modified, and can be linear or cyclized. Extracellular AIPs are detected via membrane-bound two-component sensor kinases[2,11,25,27]. Numerous Gram-negative pathogens control virulence factor production LuxI/LuxR type QS circuits. Some examples are LasI/LasR and RhlI/RhlR in *P. aeruginosa*, SmaI/SmaR in *Serratia marcescens*, VjbR (an orphan LuxR homolog) in *Brucella melitensis*, and CviI/CviR in *Chromobacterium violaceum*[24,31,35,37]. Plants inhibit the production of virulence factor of *chromobacterium violaceum* due to presence of antioxidant activity[6]. TLC profiling of plant extracts gives an impressive result that directing towards the presence of number of phytochemicals. Different phytochemicals compound gives different R_f values in different solvent system that provides a very important clue in understanding of their polarity and helps in selection of appropriate solvent system for separation of pure compounds by the help of column chromatography[9]. From the above-mentioned references, it can be concluded that due to the ethno botanical activity of the plant materials it can be consumed as food, medicine even for other purposes also. The aim of present study was to phytochemicals screening, partial separation and identification of active compounds present in these plants by TLC (thin layer chromatography) which

having bacterial quorum sensing activity as well as DPPH scavenging activity.

Collection and preparation of plant materials :

Two samples were collected from the locality of Malda which have an altitude of 17m. The collected leaves were washed well, shade dried and powdered. Ethanolic extracts of all leaves were prepared by soaking the dust in 95% EtOH using a ratio of 1 g (plant material):10 ml (EtOH) for 72 h at 30ºC with intermittent mixing. The mixture was filtered through Whatman No.1 filter paper. Then evaporate the alcohol by keeping it inside the hot air oven at a temperature less than 50ºC and the ultimate stock of plant sample is made by dissolving it in ethanol.

Bacteria and culture condition :

The strain, *C. violaceum* MTCC 2656was obtained from the Microbial Type Culture Collection Center (MTCC), IMTECH. A culture was grown in Luria- Broth (LB, Hi-Media-M575) at 37 ºC for further studies.

Anti-quorum sensing activity of plant samples :

The anti-quorum sensing activity of plant samples is performed by three different ways such as –screening of anti-quorum sensing activity of plant sample by well diffusion assay, inhibition percentage of violacein production by flask incubation assay, direct bioassay of anti-quorum compounds by thin layer chromatography (TLC).

Screening of anti-quorum sensing activity of plant sample by well diffusion assay :

The overnight grown culture *of C. violaceum* (100 µl) was poured with molten Luria Bertani agar (LB, at 50ºC) in sterile Petri plate and kept it inside the laminar air flow for 30 minutes. The agar plates were punched with the sterile cork-borer(6mm in diameter). The wells were filled with 100µl of ethanolic plant extract and kept it at 37 ºC temperature for 72 hours. QS Inhibition was detected by a halo (colourless growth signifying a failure of violacein production) surrounding the well.

Inhibition percentage (%) of violacein production by flask incubation assay :

This assay was performed in two steps flask incubation assay is done in the first step and quantification of violacein production was done in the second step. One ml of whole night growing culture was transferred to each of the ten flasks which contain 18 ml of LB broth and supplemented with 1 ml of different concentration of (100 µg/ml, 50 µg/ml, 25 µg/ml) plant extracts. For the control 1 ml of sterile distilled water is added instead of plant extract. All the flasks are kept in a rotary shaker at 150 rpm then centrifuged at 10000 rpm for 10 mints to precipitate insoluble violacein. The supernatant was discarded and 1ml of DMSO was added to the pellet, vortexed, vigorously until the violacein was completely dissolved, then centrifuged at 10000 rpm for 10 min to remove cells. The absorbance was read at 585nm and treated groups were compared with control group. The inhibition (%) is calculated by the following formula-

$$\text{Inhibition}(\%) = \frac{\text{OD585 of control} - \text{OD 585 of treated} \times 100}{\text{OD585 of control}}$$

Thin-layer chromatography for direct bioassay of anti-quorum sensing activity of plant sample :

Dried plant sample was reconstituted in ethanol in the concentration of 10mg/ml.10µl of a sample was spotted on TLC plates and eluted in the non-polar solvent hexane/ethyl acetate (3:1).After elution, the plates were dried until the solvent evaporated properly. After that, the TLC plates were overlaid with a thin film of soft nutrient agar (0.8% w/v agar). After incubation of 2 days 37°C. QSI was located as the colourless turbid circle in the purple background.

Phytochemical screening of plant materials (Qualitative and Quantitative) :

Qualitative analysis :

Qualitative analyses were performed to detect the presence of carbohydrates, protein, phenol, tannins, flavonoids, glycosides etc. by following the standard protocols[33,39].

Quantitative analysis of the constituents:

Estimation of total soluble & reducing sugar and protein present in the dried plant sample was done by following standard protocol[25].

Antioxidant activity of plant sample by DPPH free radical scavenging activity :

DPPH (2, 2-diphenylpicrylhydrazyl) free radical scavenging activity was done by different concentrations of plant extracts (0.1 ml) were put in the test tube and 2.9 ml of a methanolic solution of DPPH (0.1 mM) was added. Kept in dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank and scavenging activity was calculated using this formula-Ao=absorbance of the blank.

$$DPPH\ Scavenging\ (\%) = 100 \times (Ao - As)/Ao$$

Thin layer chromatography (TLC) :

Phytochemicals screenings by TLC were performed for detection of active compounds present in the different plant samples. For this, the dried plant sample was reconstituted in ethanol to a concentration of 10 µg/ml. Each solvent extract was subjected to TLC as per conventional screening of one dimensional ascending method using silica gel 60F254, 7X6 cm (Merck)[33].

Plant material :

The collected plant samples were identified from the herbarium specimens of the University of North Bengal. The scientific name of the plant samples is *Nasturtium officinale* R.Br. (Brassicaceae), *Amaranthus spinosus* L. (Amaranthaceae) (Fig 1).

Preparation of plant extract :

The ethanolic plant extract was made by adding ethanol in 1:10 ratio and the process was repeated for 3 times. *A. spionosus* showed higher (85%) extractive value as compared to *N. officinale* (Table 1).

Table-1. Extractive value of two samples

Name of the plant sample	Extractive value of the sample (%)
Amaranthus spinosus	85.0
Nasturtium officinale	18.5

Fig. 1. Flowering plant of a: *Nasturtium officinale* b: *Amaranthus spinosus*

Anti-quorum sensing activity of plant extract :

The anti-biofilm activity of plant sample was done by the three different ways such as agar well diffusion method, flask incubation assay and bioassay for quorum sensing inhibition by TLC. In well diffusion assay colour less zone was observed around the well without inhibiting the growth of *C. violaceum*.

In flask assay, the violacein production was inhibited by *A. spinosus* and *N. officinale* properly. A concentration dependent inhibition of violacein production was observed in case of both the extracts. In case 50 mg/ml of plant extract the inhibition (%) was more than 50 %. In higher concentration the inhibition of violacein production was higher in case of *A. Spinosa* in comparison to *N. officinale* (Fig 2).

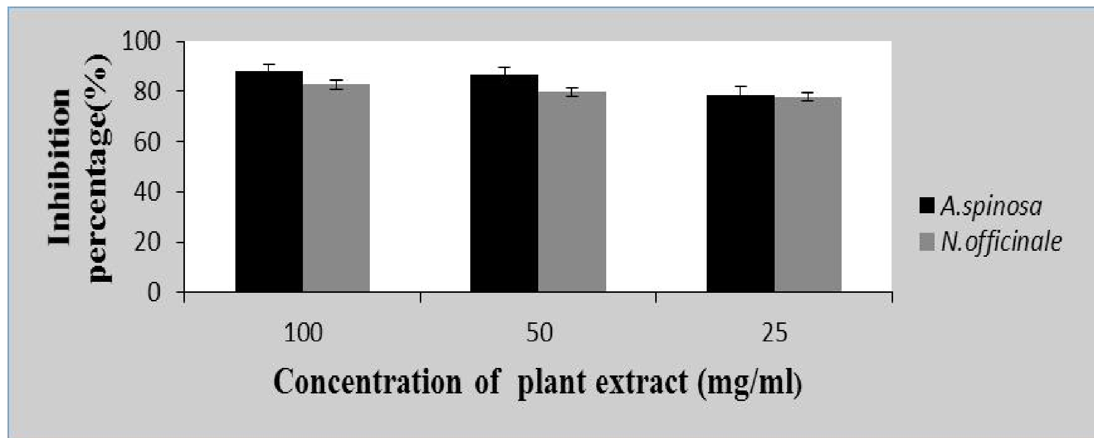
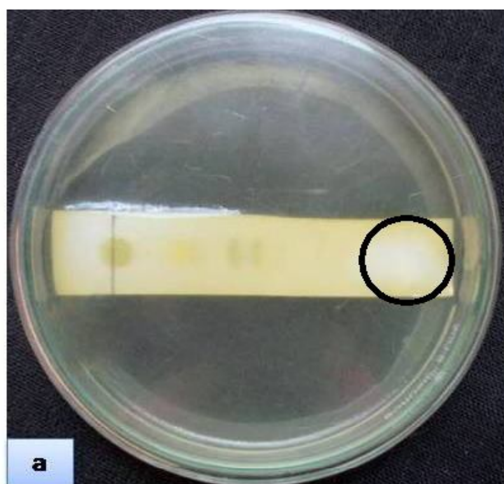


Fig. 2. Study of inhibition percentage (%) of violacein production in *A. spinosus* and *N. officinale* by flask incubation assay

The thin layer directed (TLC) bio autography was used for identifying the compounds which are responsible for the anti QS activity in *N. officinale, A. spinosus*. The zone of inhibition determined by the help of R_f values which shows two unique spots likes 0.49 and 0.51. (Fig. 3).



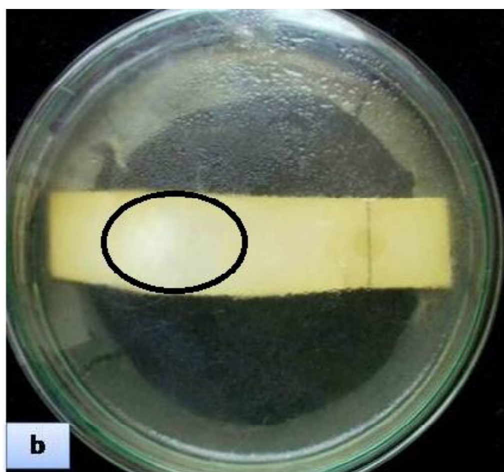


Fig. 3. TLC bioautography of two plants samples showing a clear zone on the plate (a-*N. officinale,* b-*A. spinosus*)

Phytochemical screening of the two plant samples :

The result revealed that both the sample consists of carbohydrate, protein, glycosides. In *A. spinosus* phenol was absent but flavonoids and tannin was present. Similarly, in *N.officinale* phenol was present but flavonoids and tannin is absent (Table 2).

Table-2. Phytochemical compounds detected in the two samples

Name of the compounds	*N. officinale*	*A. spinosus*
Carbohydrate	+	+
Protein	+	+
Flavonoid	_	+
Glycoside	+	+
Phenol	+	_
Tannin	_	+

Nutritional factors :

A different nutritional factor (total protein, total soluble sugar and total reducing sugar) of ethanolic extract was determined by quantitative analysis. The result showed that the amount of protein and total sugar was higher in *N. officinale,* but the amount of reducing sugar was high in *A. spinosus* (Table 3).

Antioxidant activity of plant sample by DPPH free radical scavenging activity:

DPPH is a stable free radical containing an odd electron having a characteristic absorption at 517nm. The deep purple colour usually gets decolourized when exposed to antioxidant in the solution. The result concludes that it was a dose-dependent inhibition with increasing in concentration of the plant sample (Fig 4).

Table-3. Different nutritional factors detected in two samples

Sample name	Total protein (µg/ml)	Total soluble sugar (µg/ml)	Total reducing sugar(µg/ml)
A. spinosus	348.27	37.6	63.1
N. officinale	536.19	83	19.7

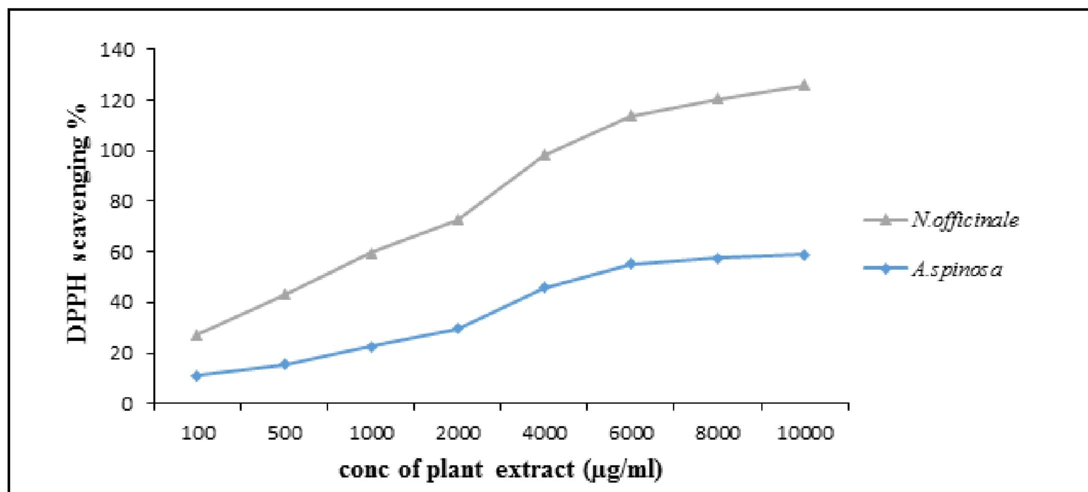


Fig. 4. Graphical representation of antioxidant activity of *N. officinale* and *A. spinosa* by DPPH scavenging method

Thin layer chromatography (TLC) :

Different types of stains were used for detection coumarins, tannin, saponins and flavonoids. Coumarin and saponins are present in both the sample. But tannin and flavonoid present only in *A.spinosus* (table 4 and Fig-5, 6).

Table-4. Different compounds detected through TLC in both the samples

Name of the compounds	*A. spinosus*	*N. officinale*
Flavonoids	+	-
Tannin	+	-
Coumarin	+	+
Saponin	+	+

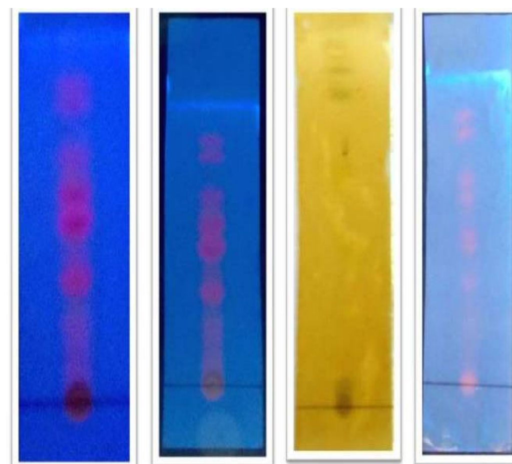


Fig. 5. TLC plates for different compounds observed under UV. A: *A.spinosus* (coumarin), b. *N. officinale* (coumarin)c. *A. spinosus* (tannin),d *A.spinosus* (flavonoid).

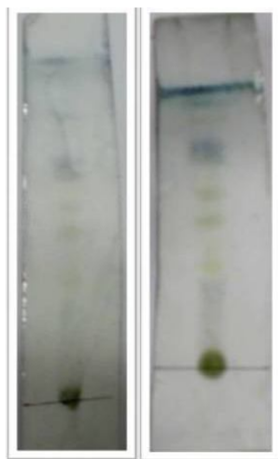


Fig. 6. TLC plates showing the presence of saponins. a. *A. spinosus*, b. *N. officinale*

The continuing emergence and spread of multidrug-resistant bacteria has led to increase use of anti pathogenic strategy to combat bacterial infections through the interruption of the quorum sensing controlled virulence factors. The inhibition of violacein production by well diffusion assay has been reported which were affected by QS-I compounds, that diffused from the well to the surrounding agar medium, fail to turn out violacein production. This type of inhibition of bacterial QS systems was reported in extracts of quite a few plants like black salsify, vanilla, pea, alfalfa and garlic[1,4,15,29]. In our experimental work, these two samples were shown inhibition (%) in flask incubation assay >80% at highest concentration and >70% at the lowest concentration. This is suggestive that both the plant samples have a broad spectrum effect in signalling of the violacein production in *C. violaceum*. The TLC directed bio autography also showed distinct two spots with R_f value 0.49 and0.51 which indicates the inhibition of quorum sensing by those two samples. The phytochemicals study and TLC of the samples have also been carried out to identify the compounds. It is difficult to comment on the active compound of the samples which responsible for the anti QS activity. It is found that the ethanolic extract of the plant samples shows presence of different phytochemicals such as protein, flavonoids, glycosides, tannin and even saponin also. This data corroborated the findings of other authors where these compounds exhibited antimicrobials activities[21]. The presence of flavonoids indicates the naturally occurring phenolic compound, with beneficial effects in the human diet as antioxidants and neutralizing free radicals[5]. Tannins are group of polymeric phenolic compound and cause local tumours. So, it is possible that both direct and indirect mechanisms may be responsible for QS interference by phytoconstituents[17]. It is proposed that anti-QS screening of plant extracts should be carefully conceded in a concentration-dependent manner and be considered as a part of an anti-infective screening strategy. Further work is necessary to identify the active compounds and their possible purification to understand the exact mechanism of action on QS controlled expression of bacterial functions as well as therapeutic efficacy.

In the present study, the two plant samples show potent anti QS activity against *C. violaceum.* These medicinal plants could manage *Chromobactrium* pathogenesis and hinder its dissemination. The presence of active compounds may be responsible for the anti QS activity of these samples. The antioxidant activity of two samples is a good source of natural compounds to prevent diseases caused due to age related diseases and free radical

scavenging. The presence of phytochemicals and the amount of nutritional factor is very high in both of the plant samples. So it can be said that it is a natural source of anti QS compounds and antioxidant compound. Further study of both of these plant extract may be lead to the creation of natural compound or drug, which shows the anti pathogenic activity.

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