Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 11, 2015

Original Article

ANTI-QUORUM SENSING ACTIVITY OF SOME COMMONLY USED TRADITIONAL INDIAN SPICES

WALLY MUTUNGWA, NARENDRANATH ALLURI, MALA MAJUMDAR*

Center for Post Graduate Studies, Jain University, 18/3, 9th main, 3rdblock, Jayanagar, Bangalore 560011, India
Email: malamajumdar51@gmail.com

Received: 31 Jul 2015 Revised and Accepted: 10 Sep 2015

ABSTRACT

Objective: To investigate the total phenolic and flavonoids contents and study the anti-quorum sensing activity of Indian spices.

Methods: The methanolic extracts of eight Indian spices were evaluated for phytochemicals and QSI (quorum sensing inhibition). QSI was determined by qualitative and quantitative violacein inhibition using *Chromobacterium violaceum*12472 as a reporter strain. Biofilm and inhibition of virulence factors viz. EPS (exo poly saccharide production), pyocyanin, proteolytic and swimming motility were assessed against clinically isolated *Pseudomonas aeruginosa*.

Results: The phytochemical screening of methanolic extract of Indian spices revealed the presence of tannins, flavonoids, terpenoids, cardiac glycosides, carbohydrates, alkaloids and phenolic compounds. Among all the spices, the total phenol (35±0.53 mg/GAE/g DW) and flavonoid (18±0.22 mg/QE/g DW) contents were found to be maximum in *Sygygium aromaticum*. *S. aromaticum* exhibited highest quorum sensing(57.63±04%) and biofilm inhibition (49.36±1.5%) at 200 mg/ml. *S. aromaticum* also showed dose dependent inhibition of virulence factors such as EPS production, pyocyanin, proteolytic and swimming motility against clinically isolated *Pseudomonas aeruginosa*. ATR-IR (Attenuated total reflectance infrared) analysis of *S. aromaticum* extract showed phyto constituents with hydroxyl, alkynes, anhydrites, alkene, nitro compounds, aromatics, esters, sulfoxide and halogen functional groups.

Conclusion: The present study promisingly revealed that *S. aromaticum* has an effective inhibition of biofilm caused by quorum sensing and virulence factors against clinically isolated *P. aeruginosa*. Biofilm prevents the entry of antibiotics, thereby developing drug resistance. The quorum quenching phyto compounds inhibit the bacterial communication and make them less virulent might be a novel non-antibiotic therapeutic system for pathogenic bacteria. Further research is necessary to identify the bioactive compound(s) responsible for the activities.

Keywords: Quorum sensing inhibition, Biofilm, Virulence factors, *Sygygium aromaticum*.

INTRODUCTION

Quorum sensing (QS), a population density dependent mechanism present in many bacteria, is mediated through auto inducers (small signal molecules) which regulate the target gene expression responsible for the phenotypes essential to pathogenicity/symbiosis leading to biofilm formation. Biofilm is the matrix of EPS serves as a protective function by reducing antibiotic efficiency thereby developing drug resistance [1]. The virulence factors produced by bacteria help to develop pathogenicity and reduce the effect of host immune system [2].

The antibiotics isolated from one bacteria being used therapeutically can independently and simultaneously induce biofilms in other bacteria by activating specific signalling pathways [3]. The antibiotics may also enhance the biofilm formation when used at sub MIC (minimum inhibitory concentration) [4]. Conventional antibiotics possess broad range efficacy via toxic or growth-inhibitory effects on target organisms. The excessive and indiscriminate usage of antibiotics has emerged in MDR (multiple drug resistant) bacterial strains which have become a growing concern worldwide [5]. Due to MDR pathogens million people die annually of infectious diseases. Not only do most antibiotic treatments fail but also an immune system has difficulties to eliminate bacteria in a biofilm [6].

Since ancient times, Indian spices have been used as medicine due to their beneficial effects against infectious diseases [7]. Recent research has revealed that few spices have the property of modulating bacterial QS system, thereby reducing the virulence [8-10]. The plant compounds usually target the bacterial QS system via different ways by stopping the signalling molecules from being synthesized, degrading the signalling molecules and/or targeting the signal receptor [11, 12]. In this scenario, the present study was aimed to investigate the effect of commonly used Indian spices on the QS and virulence factors inhibition.

MATERIALS AND METHODS

Chemicals and reagents

Azocasein, Gallic acids and Quercetin were purchased from Sigma-Aldrich (St. Louis, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck, Germany. All other chemicals used in the study were of analytical grade.

Sample collection and extraction

The spices (*Syzygium aromaticum, Trigonella foenum-graecum, Cariandrum sativum, Capsicum annuum, Brassica juncea, Pipper nigrum, Papaver somniferum* and *Nigella sativa*) were collected locally in and around Bangalore, Karnataka, India. The plant materials were identified by Dr. S. B. Sullia, Botanist and Microbiologist, Jain University. The materials were cleaned, dried and powdered. The powdered samples were extracted with methanol at 1:10 (w/v) concentrations by using Soxhlet apparatus. The extract was filtered through Whatman No.1 filter paper and the filtrate was concentrated by rotary evaporator, stored at 4 °C and used for further studies.

Strains and culture condition

Chromobacterium violaceum 12742 was procured from MTCC, Chandigarh and *Pseudomonas aeruginosa*, a clinical isolate was collected from Bhagawan Mahaveer Jain Hospital, Bangalore. The cultures were maintained in the LB (Luria Bertani) medium at regular intervals for further studies.

Phytochemicals screening

The extracts were subjected to the qualitative phytochemical screening according to the standard procedure [13].

Total phenol and flavonoid determination

Total phenol content was estimated using Folin-ciocalteau reagent according to Mc Donald *et al.*[14]and Gallic acid were used as

standard for calibration curve. The total flavonoid content was quantified according to the standard procedure [15]. Quercetin was used as standard for calibration curve.

Qualitative disc diffusion assay

The disc diffusion method was employed to detect the QSI activity of the spices extracts (50,100, 150 and 200 mg/ml) and the clear zone of inhibition around the disc was measured [16].

Quantitative violacein inhibition assay

Quantitative evaluation of QSI activity of the spices was carried out based on their ability to inhibit the production of purple pigment violacein by *C. violaceum* and pigment production were quantified at 585 nm using UV-Vis spectrophotometer [7].

Inhibition of biofilm formation and *in situ* microscopic observation

Among all the tested spices *S. aromaticum* shown best activity analysed for further studies. The effect of *S. aromaticum* extracts on the biofilm formation was determined by quantifying the biomass through microtiter plate assay [17]. The clinical pathogen was incubated in 96 well plates in the presence and absence of extracts for 16 h without agitation. The free-floating plank tonic cell was removed, washed with sterile water and stained with 0.2% crystal violet solution for 15 min. The excess crystal violet was discarded, wells were filled with 95% ethanol and absorbance was measured at 650 nm using a UV-visible spectrophotometer. For *in situ* microscopic observation of biofilm the clinical pathogen was incubated for 16 h in 6 well plates having cover glass of one cm^2 along with and without *S. aromaticum* extract. The free-floating plank tonic cell was removed, washed with sterile water and stained with 0.2 % crystal violet solution for 15 min. The adhered biofilms were observed under light microscopy [17].

Effect of extracts on virulence factors

Extraction and quantification of EPS

The clinical pathogen was grown in the presence and absence of *S. aromaticum* extract for 16 h. The incubated culture was centrifuged and the pellet was suspended in 50 ml of high-salt buffer (10 mM KPO_4, pH 7. 5 mM NaCl, 2.5 mM $MgSO_4$). The cells were removed by centrifugation and dislodged EPS was precipitated by adding three volumes of ethanol. The resulting precipitate was re suspended in an appropriate volume of double distilled water and stored at-20 °C. The extracted EPS was quantified using phenol-sulphuric acid method and absorbance was measured at 490 nm [18].

Azocasein-degrading proteolytic activity

Proteolytic activity of pathogenic bacteria was determined using Azocasein as the substrate. 150 µl of both treated and untreated pathogenic culture supernatants with *S. aromaticum* was added to one ml of 0.3% Azocasein in 0.05 M Tris-HC1 and 0.5 mM $CaCl_2$ (pH 7.5) and was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 0.5 ml trichloroacetic acid (l0%) followed by centrifugation and the absorbance was measured at 400 nm [19].

Pyocyanin quantification assay

The pyocyanin quantification assay was performed by inoculating the pathogenic bacteria into the pyocyanin broth (peptone–20g, $MgCl_2$–1.4 g, K_2SO_4–1.0 g/l, and pH7.4) in the presence and absence of *S. aromaticum* extracts. After 24 h of incubation, the cells were removed by centrifugation and the broth was extracted with equal volume of chloroform and then re-extracted with one ml of 0.2 N HCl. The absorbance of the solution was measured at 520 nm [20].

Swimming assay

For swimming assay, the overnight cultures of pathogenic bacteria were point inoculated at the center of the plates containing 1% tryptone, 0.5% NaCl and 0.3% agar with and without *S. aromaticum* extracts. Plates were incubated for 16 h at 37 °c and the swimming migration was measured [20].

ATR-IR analysis of *S. aromaticum*

S. aromaticum was subjected to ATR-IR (Bruker Alpha ECO-ATR spectrometer) analysis for the functional groups. A small amount of sample was placed directly on the germanium piece of the IR spectrometer with constant pressure and wave number ranged from 4000 cm^{-1} to 675 cm^{-1}. The characteristic peaks were determined according to their functional groups [21].

Statistical analysis

All the experiments were carried out in triplicates. Results were expressed as the mean±S. E (Standard Error) of three independent experiments (n=3).

RESULTS

Phytochemical analysis

The phytochemical screening of methanolic extract of Indian spices revealed the presence of tannins, flavonoids, terpenoids, cardiac glycosides, carbohydrates, alkaloids and phenolic compounds (table 1). Among all the spices, the total phenol (35±0.53 mg/GAE/g dry weight) and flavonoid (18±0.22 mg/QE/g dry weight) contents were found to be maximum in *S. aromaticum* (table 2).

Effect of Indian spices on QSI

The qualitative (6±0.3 mm) and quantitative (57.63±0.4%) violacein inhibition of spices revealed that *S. aromaticum* showed the highest inhibition at 200 mg/ml followed by *C. annuum* and *B. juncea* respectively (table 2).

Effect of *S. aromaticum* on *P. aeruginosa*

S. aromaticum extract exhibited maximum biofilm inhibition at 200 mg/ml (49.36±1.5%). *In situ* microscopic observation of the biofilm showed a thick layer of biofilm in control cover slips and *S. aromaticum* treated one exhibited concentration dependent biofilm inhibition against *P. aeruginosa.* The *S. aromaticum* showed significant effect on the virulence factors viz. EPS, pyocyanin, proteolytic and swimming motility inhibition of *P. aeruginosa* compared to un treated one.

Spectrometric analysis of the extracted EPS revealed that the concentration of EPS decreased with increasing concentration of *S. aromaticum*. The test extract exhibited 58.06±0.74% inhibition of EPS production of *P. aeruginosa* at 200 mg/ml. The tested extract showed maximum pyocyanin inhibition (60.15±0.43%) and the proteolytic activity (45±0.6%) at 200 mg/ml(table 2). It also exhibited significant swimming motility inhibition of *P. aeruginosa* at 200 mg/ml (fig. 1).

Table 1: Qualitative phytochemical screening of Indian spices

Test group	Phenols	Flavonoid	Alkaloids	Terpenoids	Saponins	Tannins	Cardiac glycoside	Carbohydrate
B. juncea	+	+	-	+	-	+	+	+
C. annuum	+	+	-	+	-	+	+	+
C. sativum	+	+	-	+	-	+	+	+
N. sativa	+	+	+	-	-	-	+	+
P. nigrum	+	+	-	+	-	+	+	+
P. somniferum	+	+	+	+	-	+	+	+
S. aromaticum	+	+	-	+	-	+	+	+
T. Foenum graecum	+	+	-	+	-	+	+	+

+: detected; -: not detected

Table 2: Total phenolic, total flavonoid contents and QSI of Indian spices

Plant Extract	Total phenolic content (mg/GAE/g DW)	Total flavonoid content (mg/QE/g DW)	Qualitative QSI (nm)at 200 mg/ml	Quantitative QSI (%) at 200 mg/ml
B. juncea	17.1±0.34	3±0.43	4±0.7	34.75±0.5
C. annuum	10±0.24	2.±0.57	5±0.4	42.37±0.6
C. sativum	8.5±0.38	5±0.43	---	---
N. sativa	17.9±0.53	7±0.67	---	---
P. nigrum	17.7±0.62	11±0.71	---	---
P. somniferum	2.6±0.22	1±0.63	---	---
S. aromaticum	35±0.53	18±0.22	6±0.3	57.63±0.4
T. Foenum graecum	7.5±0.45	2.5±0.47	---	---

Each value in the table is represented as mean±SD (n=3). mg GAE/gm DW**:** milligram gallic acid equivalent per gram dry weight mgQE/gm DW: milligram quercetin equivalent per gram dry weight

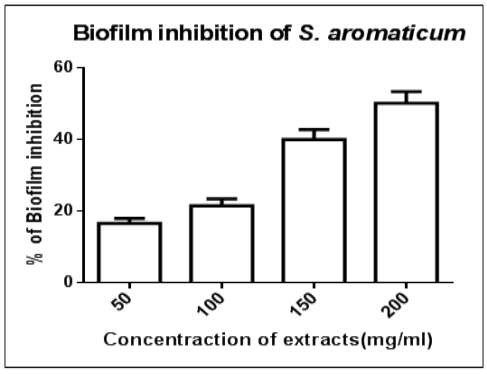

Fig. 1: Biofilm inhibition of *S. aromaticum*
Each value is expressed as mean±SE (Standard Error) (n=3)

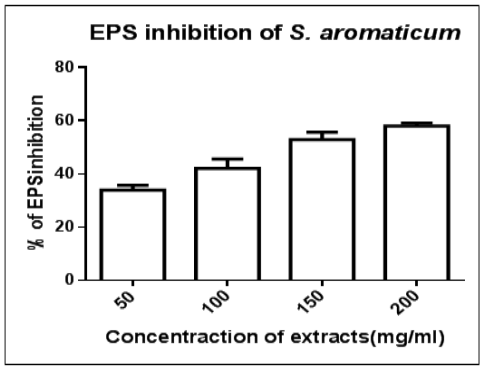

Fig. 2: EPS inhibition of *S. aromaticum*
Each value is expressed as mean±SE (Standard Error) (n=3)

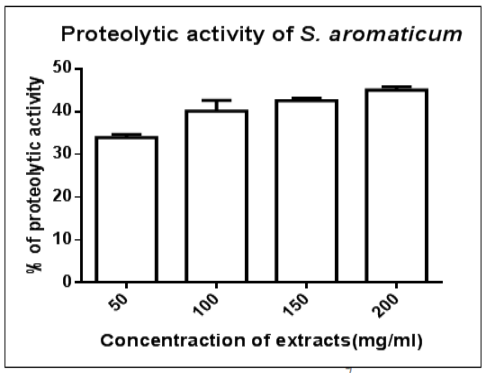

Fig. 3: Proteolytic activity of *S. aromaticum*
Each value is expressed as mean±SE (Standard Error) (n=3)

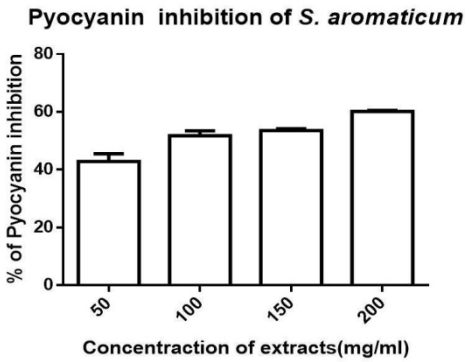

Fig. 4: Pyocyanin inhibition of *S. aromaticum*
Each value is expressed as mean±SE (Standard Error) (n=3)

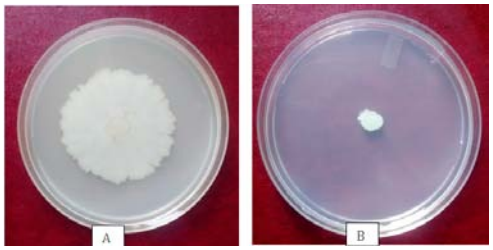

Fig. 5: Swimming inhibition of *S. aromaticum* on *P. aeruginosa*
Where; A= Control, B= *S. aromaticum* at 200 mg/ml

ATR-IR analysis of *S. aromaticum*

ATR-IR analysis of *S. aromaticum* confirmed the presence of hydroxyl, alkynes, anhydrites, alkene, nitro, aromatics, esters, sulfoxide and halogen functional groups with major peaks at3459.059, 2965.49, 1764.202, 1625.513, 1501.101, 1260.435, 1201.289, 1038.126and 611.8618 cm^{-1} respectively (fig. 6).

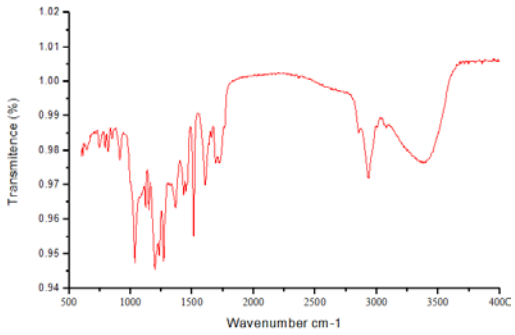

Fig. 6: ATR-IR analysis of TLC bioactive fraction of *S. aromaticum*

DISCUSSION

Ayurveda is originated in India and known as the oldest traditional systems medicine with more than2000 therapeutic plant species. Screening of medicinal plants for their bioactivity is important since traditional medicines are used as complementary and alternative medicine widely throughout the world. [22]. Herbs and spices are common ingredients as flavouring and preservative in food preparation among the Indians. These are also used as preventive and curative medicines. Agaoglu *et al.* [9] reported that the antibacterial activity of spices *Cinnamomum zeylanicum*, *Syzygium aromaticum* and *Cuminum cyminum* against Gram negative and Gram positive bacteria.

In the current study, the phyto chemical screening of spices revealed the presence of tannins, flavonoids, terpenoids, cardiac glycosides, carbohydrates, alkaloids and phenolic compounds. Quantitatively total phenolic contents are prevalent in all the eight Indian spices compared to other phyto chemicals ranging from 2.6-35 mg/GAE/g DW. Among all the extracts, *S. aromaticum* possesses highest total phenolic and flavonoid contents (table 2).

Very few reports are available related to the quorum sensing inhibition of Indian spices [16, 17, 22]. In the present study, *S. aromaticum* exhibited highest quorum sensing inhibition in comparison to all the tested spices and analysed for further studies. *S. aromaticum* showed concentration dependent violacein pigment inhibition on *C. Violaceum* which is in accordance with Khan *et al.* [16]; Krishnan *et al.* [22]. The *in situ* microscopic observation of biofilm inhibition showed dose dependent inhibition of biofilm formation [17]. The EPS formation was inhibited by *S. aromaticum* which indicates that the bacterium was unable to attach to host for the formation of biofilms and susceptible to host immune system leading to the eradication of the plankton bacterium. The virulence factors viz. pyocyanin, proteolytic and swimming motility helps the bacterium to promote the pathogenicity and acts against the host immune system. The *S. aromaticum* showed significant inhibition of above virulence factors, thereby inhibiting spreading of the disease and thus helps the host immune system. The above findings are in accordance to the earlier investigations [17, 22, 23]. The ATR-IR analysis of spectra showed the presence of alkanes, alkynes, aldehydes, aromatics, carboxylic acids and halogen groups.

CONCLUSION

It is concluded that *S. aromaticum* exhibited an effective inhibition of biofilm and virulence factors against clinically isolated *P. aeruginosa*. The ATR-IR analysis of *S. aromaticum* showed the presence of phyto constituents with functional groups which might be responsible for the quorum sensing, biofilm and virulence factors inhibitory activities of *S. aromaticum* .

ACKNOWLEDGEMENT

The authors are grateful to the Jain University for providing infrastructure for carrying out the research work. We thank Dr. Geetha V Bhat, Microbiologist and Infection control officer, Bhagwan Mahaveer Jain Hospital, Bengaluru for providing *Pseudomonas aeruginosa* strain. We also thank Dr. Geetha Balakrishna, Director, CNMS, Jain University, for ATR-IR spectra analysis.

CONFLICT OF INTERESTS

Declared None

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