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Extraction of Sulforaphane from Broccoli and To Analyze Its Effect on Biofilm Formation as Quorum Sensing Inhibitor

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ABSTRACT: Isothiocyanates (ITCs) are natural plant products generated by the enzymatic hydrolysis of glucosinolates found in Brassicaceae vegetables. These natural sulfur compounds are known for their anti-cancerous properties as well as their antimicrobial properties. In this study, sulforaphane from broccoli was extracted and its activity against the common opportunistic pathogen *P. aeruginosa* was carried out. MIC of sulforaphane against *P. aeruginosa* was found out to be 8mcg. Sulforaphane activity was checked against pigment (pyocyanin) production and biofilm formation by *P.aeruginosa*. Both of these characteristics are a direct result of QS genes of *P.aeruginosa*. In this study, *P.aeruginosa* was targeted owing to its notorious ability to possess antibiotic resistant genes, which can create havoc in hospitals or immunocompromised individuals. In addition to the antimicrobial activity, sulforaphane when compared with anti-biotics against *P.aeruginosa* showed appreciable results. Therefore, sulforaphane appears to be a promising compound with anti-biofilm properties that may play an important role in preventing colonization of *P. aeruginosa* inside susceptible patients.

KEYWORDS: Anti-biotic resistance, QSI, *P. aeruginosa*, Isothiocyanates, Sulforaphane.

I. INTRODUCTION

Microbes co-habit our planet and are engaged in a struggle for survival though on a microscopic scale. This endeavour allows them to develop and devise means for survival and proliferation. One such strategy is the formation of biofilms leading to establishment of a protected community. Such multi-communities may consist of harmful and pathogenic microbes, and they may cause economic problems and threats to human health. Biofilms are formed when microorganisms are typically attached to support surfaces. Biofilm-associated cells are sessile and differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down-regulation of specific genes. Biofilm formation is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. Development of strategies to control or prevent biofilms requires a thorough understanding of the biofilm development process. [2] Biofilms have great importance for public health because of their role in certain infectious diseases and their importance in a variety of device-related infections. Because many antibiotics are unable to eradicate dense biofilms, much work is required to devise ways to prevent their occurrence and clear them from the host. [6] The role of QS is evident in the formation of dental plaque, the most complex and significant biofilm related to human health. [10] Biofilm-associated microorganisms have been shown to be associated with several human diseases, such as native valve endocarditis and cystic fibrosis, and to colonize a wide variety of medical devices. [9]

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II. RELATED WORK

In recent decades, there has been a strong rise in interest and awareness regarding the personal control of our health and prevention of disease, especially in the area of nutrition. In this respect, a well-studied group of vegetables that has raised significant interest which includes cruciferous vegetables such as broccoli, Brussels sprouts, cabbage, cauliflower, kohlrabi and kale. The beneficial properties of these vegetables have mostly been attributed to secondary metabolites known as glucosinolates and their enzymatic hydrolysis products, isothiocyanates. [4] Sulforaphane belongs to a group of phytochemicals, or disease-fighting compounds in plant foods, known as the isothiocyanates. Along with related phytochemicals, it helps to prevent against the development of cancer. Sulforaphane prevents certain enzymes from activating cancer-causing agents in the body and increases the body's production of other enzymes that clean carcinogens out of the system before they can damage cells. According to sources such as the [Breast Cancer Research Program], Sulforaphane is produced in cruciferous vegetable plants only when two enzymes in separate "sacs" react, myrosinase and glucoraphanin. Broccoli sprouts are the richest food source of glucoraphanin, the precursor to sulforaphane, or SFN, also known as glucoraphanin sulforaphane. The enzyme myrosinase (thioglucosideglucosylhydrolase) transforms glucoraphanin (β -thioglucoside-N-hydroxysulfate) into the bioactive compound sulforaphane upon damaging the plant, such as chewing or chopping. [4]

Three-day old broccoli sprouts are concentrated sources of this phytochemical, offering 10 to 100 times more of it, by weight, than mature broccoli plants or cauliflower, according to research published in September 1997 in the ["Proceedings of the National Academy of Sciences."] A 1-ounce serving provides 73 milligrams of sulforaphane glucosinolate. Per 100-gram serving, broccoli sprouts offer approximately 250 milligrams. Glucosinolates are water-soluble compounds that are leached into cooking water. These phytochemicals are easily destroyed. Boiling cruciferous vegetables for just 9 to 15 minutes decreases total glucosinolate content by 18 to 59 percent, according to research published in September 2003 in the "[British Journal of Nutrition]."

With respect to treatment, a novel antimicrobial strategy, which is expected to transcend problems with selective pressures for antibiotic resistance, is to interrupt bacterial communication, also known as quorum sensing (QS), by means of signal antagonists, the so-called QS inhibitors (QSIs). Furthermore, QSI agents offer a potential solution to the deficiencies associated with use of traditional antibiotics to treat infections caused by bacterial biofilms and multidrug-resistant bacteria. [7]

QS in *P. aeruginosa*

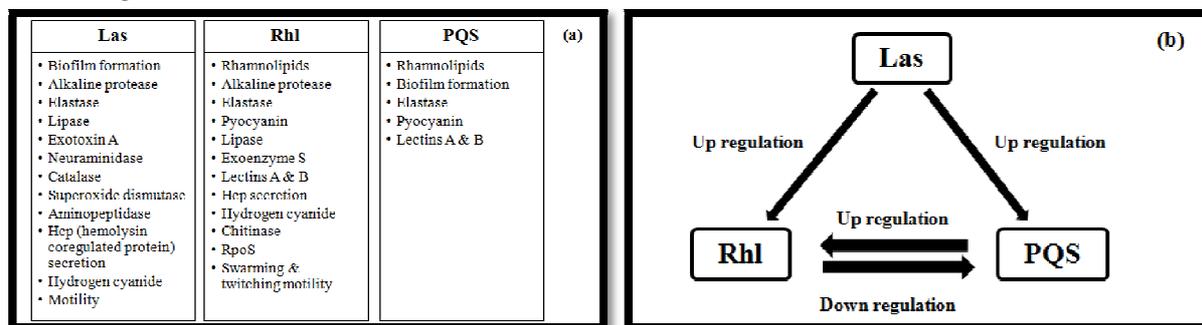


Fig 1. (a) The regulatory functions of Las, Rhl and PQS systems of *P. aeruginosa* and (b) The interlinked regulatory circuit of Las, Rhl, and PQS. [3]

III. MATERIALS AND METHODS

1. Extraction of sulforaphane from broccoli by using ethyl acetate and estimation of sulforaphane content.

Extraction of sulforaphane from broccoli by using ethyl acetate. [8]

Broccoli was dried by freeze-drying at -20°C for about 40-45 days, and then the dried sample was crushed into powder mechanically. 0.50 g sample powder was accurately weighed and homogenized with 10.0 ml phosphate

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buffer (K_2HPO_4 and KH_2PO_4 , 0.1 mol, pH 7.0), shaking for 1 hour in a flask, and then it was extracted by 30 ml ethyl acetate. All the solutions were transferred to 50 ml tube, and then it was centrifuged for 10 min at 6,000rpm. The supernatant was collected and the residual liquid was extracted by ethyl acetate until the third time using the same process. Then all collected supernatant was evaporated by at $35^\circ C$ in water bath. The dry residue was then dissolved in 10 ml methanol, being filtered through No. 0.22 μm nylon filter (D 13 mm) and stored at $-20^\circ C$ for determination. In the study, different pH values, reaction time (hour) and the ratio of buffer to materials (ml/g) were used to determine the optimum values. They are individually as follows:

pH value:- 7.0, 7.5, Reaction times (hour):- 1.0 hr., 2.0 hrs,

2. Determination of functional group by FTIR and to qualitatively confirm the presence of sulforaphane in the powder.

FTIR analysis was carried out at low resolution to detect the presence of functional groups in the sulforaphane molecule. Sulforaphane has isothiocyanate ($N=C=S$) group which is characteristic for the sulforaphane. In addition, it also has CH_3 and CH_2 both of which can be detected by the FTIR technique. Thus, the presence of these three groups in the powder will ultimately result in the confirmation of sulforaphane molecule in the powder.

3. Determination of Minimum Inhibitory Concentration (MIC) by tube method.

Pseudomonas aeruginosa was grown in Cetrimide broth for 24 hours at $37^\circ C$. The determination of the MIC of sulforaphane was carried out using the working stock i.e 50mcg of sulforaphane diluted in DMSO. The diluent used was sterile LB broth. Diluent without stock (sulforaphane) was taken as media control, set of 2 tubes i.e positive and negative control were run simultaneously. The tubes were incubated for 24 hours at $37^\circ C$. Standard range for MIC determination was 2mcg-20mcg of sulforaphane.

As 1-ounce serving provides 73 milligrams of sulforaphane glucosinolate. Per 100-gram serving, broccoli sprouts offer approximately 250 milligrams (British Journal of Nutrition).

1 ounce corresponds to 28.34g, thus
 $28.34g = 73mg = 0.073g$ of sulforaphane
 $75g = ?$

Therefore, $75g = 0.19g$ of sulforaphane.

Preparation of stock:-

0.19g
 1:1000 (0.01g + 10ml)

$$0.00019g = 0.19mg * 1000 = 190mcg$$

For 50mcg of sulforaphane stock:-
 RT/G formula is used in which,
 $R = 50mcg$, $T = 30ml$, $G = 190mcg$.

Calculation for working solution:-

$$50mcg * 30 / 190 = 150 / 19 = 7.89 = 7.9 + 22.1$$

(Stock) (Diluent)

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Standard Range:- 2mcg- 20mcg of sulforaphane.

Conc. of Stock (mg/ml)	Volume of stock (ml)	Volume of diluent (ml)	Total Volume (ml)	Volume of Culture (ml)
2	0.16	3.84	4.0	1.0
4	0.32	3.68	4.0	1.0
6	0.48	3.52	4.0	1.0
8	0.64	3.36	4.0	1.0
10	0.80	3.20	4.0	1.0
12	0.96	3.04	4.0	1.0
14	1.12	2.88	4.0	1.0
16	1.28	2.72	4.0	1.0
18	1.44	2.56	4.0	1.0
20	1.6	2.40	4.0	1.0
PC	-	4.00	4.0	1.0
NC	4.0	-	4.0	1.0
MC	4.0	-	4.0	-

Table 1. MIC PROTOCOL TABLE

KEY:-

1. Working Solution: - 50mcg of Sulforaphane in DMSO.
2. Working Range: - 2-20mcg of Sulforaphane.
3. Diluent: - Sterile LB broth.

4. Pyocyanin production Assay / Effect of sulforaphane on pigment production [4]

2 mL of chloroform was added to 2 mL of culture supernatant, taken from 23 hours old cultures grown in the presence of probe (sulforaphane) or only LB medium with 0.1% DMSO as control. After extraction, 1 mL of the chloroform layer was transferred to a fresh tube and mixed with 300 µL of 0.2 N HCl. After centrifugation, the aqueous (top) layer was separated and its absorption was measured at 520 nm. Thus two tubes (Control and Test) were analyzed by colorimetric method. Furthermore, the tubes were compared at OD₅₂₀ nm.

5. Effect of sulforaphane on biofilm [5]

Crystal Violet assay: This is a quantitative method for biofilm detection. A loopful of test organism was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. Tubes were incubated at 37°C for 24 hours. After incubation, tubes were decanted and washed with sterile saline and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with D/W. De-stained with 4ml of ethanol by incubating for 5mins at room temperature. The amount of biofilm formed was then detected colorimetrically at 660nm.

Detection of biofilm formation by using microscopy: Briefly, two sterile big petri plates were used to grow biofilm. Six glass slides were placed in the two sterile big petri plates and daily 200ml sterile trypticase soy broth with 1% glucose was added to both control and test labeled petri plate. After incubation at 37°C for 24 hours, the previous day media was discarded and fresh media was added. This was carried on for four consecutive days and on the fifth day, the slides were washed with sterile saline and stained with CV. The entire protocol was carried out aseptically.

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6. Comparing the potency of sulforaphane v/s Antibiotic against *P. aeruginosa*

Agar well-diffusion method: [1]

For this method, Mueller-Hinton agar plates were used. Agar surface of each plate is to be streaked by a sterile cotton swab with the reference bacterial strain. Then punch agar plate with a sterile cork borer of 6 mm size and then pour 100 μL of sulforaphane with micropipette in the bore. Allow plates to stand for 30 mins. Then incubated at 37°C for 48 hrs. Zone of inhibition (mm) of the bacterial strains against sulforaphane by Agar well diffusion method by standard protocol. A standard method of National Committee for Clinical Laboratory Standards NCCLS.

IV. RESULTS

1. Extraction of sulforaphane from broccoli by using ethyl acetate and estimation of sulforaphane content.

After extraction of sulforaphane from broccoli, powdered residue was obtained which was then dissolved in DMSO and was further used as a stock to carry out procedures like MIC, Pigment Production Assay, Biofilm Assay and to compare its efficiency against various antibiotics.

The powdered residue was then analyzed by FTIR method to confirm the presence of isothiocyanate ($\text{N}=\text{C}=\text{S}$) group of sulforaphane.

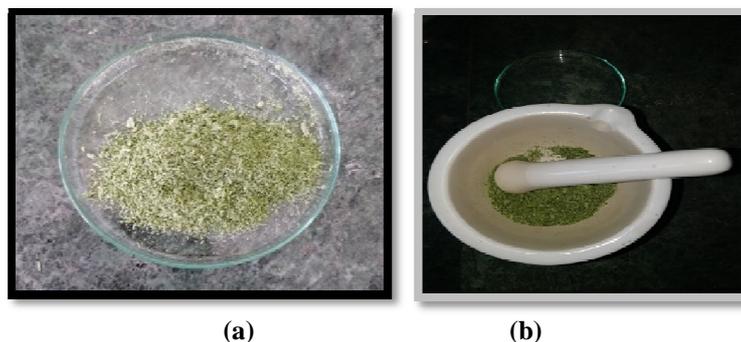


Fig 1. (a) Freeze dried florets of broccoli after 45 days at -20°C,

(b) Maceration of dried florets of broccoli,

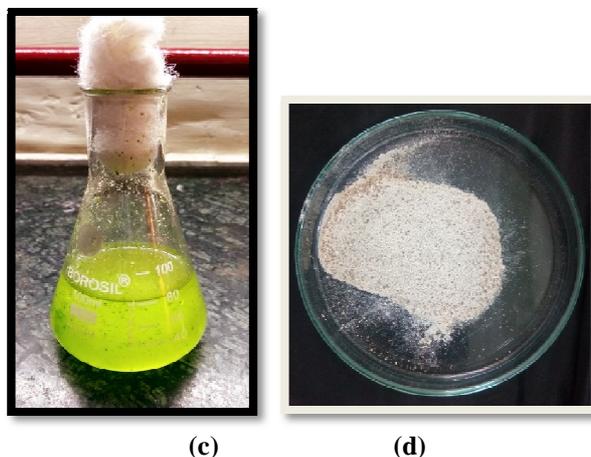


Fig 1. (c) Ethyl acetate extraction,
(d) Sulforaphane powder (yield obtained after repeated extraction).

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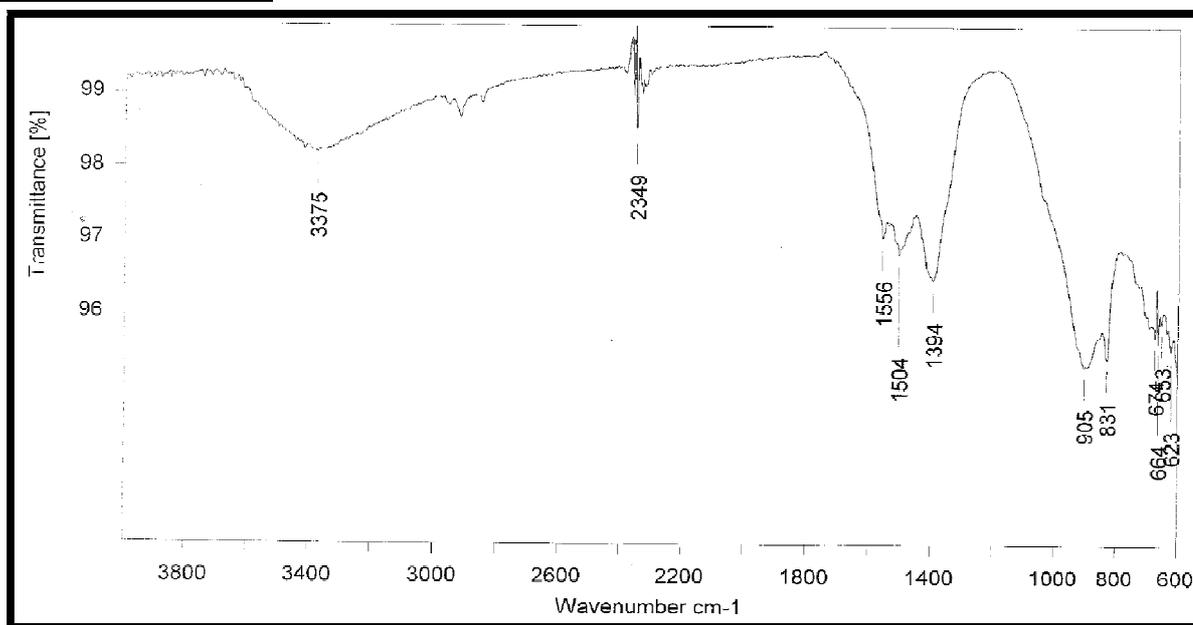
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The powder obtained after extraction was stored in cold conditions. For qualitative analysis of the powder, FTIR analysis was carried out. Since FTIR, method detects specific functional group, the detection of isothiocyanate (N=C=S) group of sulforaphane in the powder will thus confirm the presence of sulforaphane molecule.

50mcg stock of sulforaphane was prepared by using DMSO as diluent. The stock was further used to prepare a standard range of 2-20mcg of sulforaphane for checking its MIC, so that further methods can be carried out by using MIC of sulforaphane to check its anti- pigment and anti- biofilm activity.

2. Determination of functional group by FTIR Spectroscopy and to qualitatively confirm the presence of sulforaphane in the powder.



Graph 1. FTIR GRAPH

Spektrometer: Alpha

Auflösung: 4

Messzubehör: Instrument type and/or accessory

Anzahl der scans: 24

Wave Number cm^{-1}	Standard Range cm^{-1}	Functional Group	Mode of vibration	Intensity
2349	2500-2000	C=N Cyanide	Stretch	Strong
1394	1450-1375	CH ₃ Methyl	Bend	Medium
1504	1480-1440	CH ₂ Methylene	Stretch	Medium

Table 1. FTIR TABLE

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From the FTIR graph, specific values were obtained which corresponded to different functional group. Sulfur was not detected since low resolution FTIR was used, hence only cyanide (CN⁻) group was detected which showed absorbance at 2349. Since this value lies in the standard range i.e. 2500-2000 for cyanide group. Thus, the presence of CN group confirms the presence of sulforaphane in the powder. The mode of vibration observed was stretch.

Furthermore, the presence of CH₃ and CH₂ (1394 and 1504) respectively confirms the presence of sulforaphane in the powder. Both these groups show medium intensity in the standard range. The mode of vibration observed was bend for CH₃ and stretch for CH₂.

As these three groups are present in sulforaphane, molecule it is quite clear that SFN is present in the powder extracted from broccoli. (Refer Graph 1 and Table 1)

3. Determination of Minimum Inhibitory Concentration (MIC) by tube method.

As the tubes were originally muddy (unclear) before incubation, thus justifying the MIC proved to be difficult. Therefore, after 24 hrs incubation, spot inoculation of the originally turbid tubes (8mcg-20mcg) was performed on MH agar and the MIC obtained for sulforaphane was found out to be **8mcg**. (Refer Table 1, Fig 1 and 2)

Conc. of Stock (mg/ml)	Growth observed
2	+
4	+
6	+
8	-
10	-
12	-
14	-
16	-
18	-
20	-
PC	+
NC	-
MC	-

Table 1. MIC RESULTS TABLE

KEY:-(+): Turbidity observed. (-): No Turbidity observed.



Fig 1. MIC of sulforaphane (8mcg)

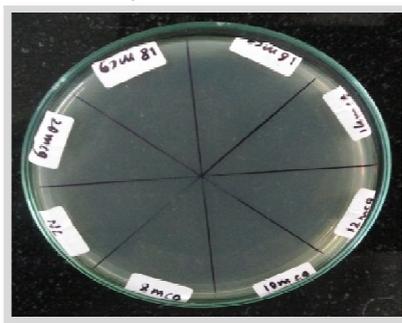


Fig 2. Confirmation of MIC of sulforaphane (8mcg) by (Miles and Misra technique) after spot inoculating on MH agar from tubes labelled 8mcg-20mcg.

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4. Pyocyanin production Assay / Effect of sulforaphane on pigment production.

After performing Pyocyanin Production Assay, which is to check the effect of sulforaphane against pigment production by *P. aeruginosa*, turbidity was obtained which was determined colorimetrically at 520nm. After comparing the values of both control and the test tubes stark difference was obtained which clearly indicates that sulforaphane inhibits the pigment production. (Refer Graph 1, Table 1 and Fig 1)

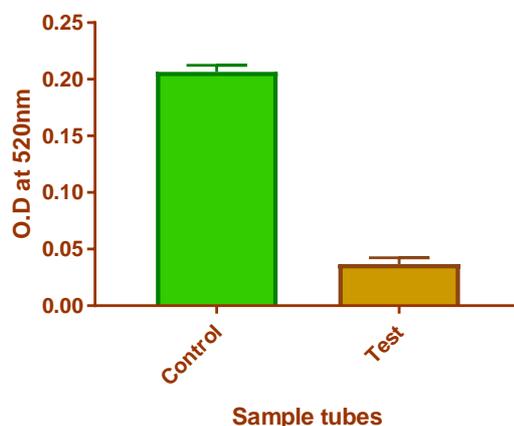


(a) Control
(b) Test

Sample Tubes	O.D at 520 nm
CONTROL	0.21
	0.21
	0.20
TEST	0.04
	0.03
	0.04

Table 1. Anti- pyocyanin pigment results table

Pyocyanin Pigment Production Assay****



Graph 1. Pyocyanin Pigment Production Assay (p value : <0.0001)

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5. Anti-biofilm activity: (Effect of sulforaphane on biofilm),(Hassan, A., et al., 2011)

1. By Crystal violet Tube Assay method (Quantitative)

After performing Biofilm Assay, which is to check the effect of sulforaphane against biofilm production by *P. aeruginosa*, violet color was obtained which was determined colorimetrically at 660nm. After comparing the values of both control and the test tubes stark difference was obtained, which clearly indicates that sulforaphane inhibits the biofilm formation. (Refer Graph 1, Table 1, Fig 1)

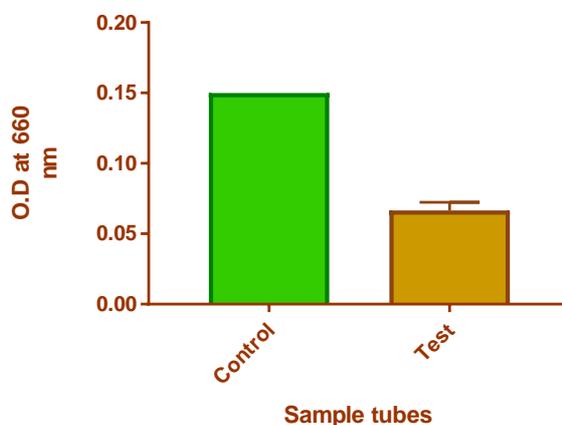


Fig 1. CV Assay (Quantitative Tube Method) for determining reduction in biofilm formation by *P. aeruginosa*.

Sample Tubes	O.D at 660 nm
CONTROL	0.15
	0.15
	0.15
TEST	0.07
	0.06
	0.07

Table 1. Anti- Biofilm Results Table

Anti-biofilm Activity by Crystal Voilet Assay



Graph 1. Anti-biofilm Activity by Crystal Violet Assay (p value : <0.0001)

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2. By CV staining and Gram staining (using microscopy)

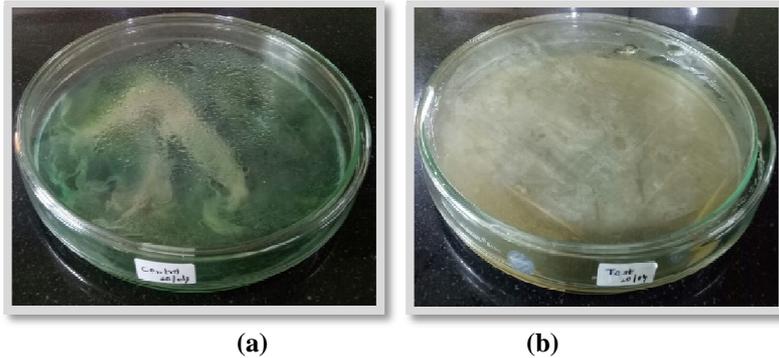
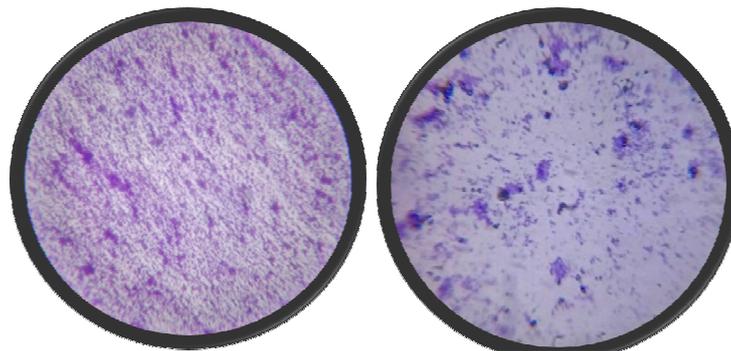


Fig 1. (a) Control
(b) Test



CONTROL Fig 2. (a) CV stain TEST

Microscopically biofilm reduction was determined and the results were in accordance with the results obtained by Quantitative CV Assay method. Thus, the results indicate that SFN is a biofilm inhibitor of *P. aeruginosa* and can be established as a QSI. (Refer Fig 2.)

6. Sensitivity Test of sulforaphane v/s Antibiotic against *P. aeruginosa*. Agar well-diffusion method:

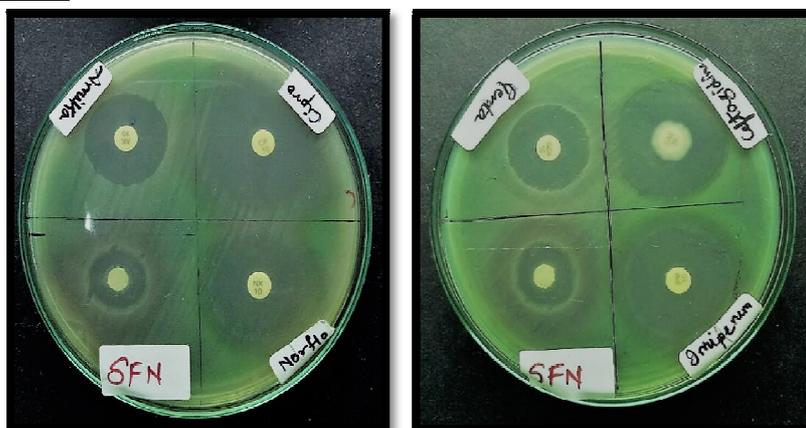


Fig 1. AST v/s Sulforaphane against *P. aeruginosa*

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ANTIBIOTIC	Concentration (in mcg)	Zone of Inhibition (in mm)
AMIKACIN	10	21
CIPROFLOXACIN	10	34
NORFLOXACIN	10	23
IMIPENEM	10	29
CEFTAZIDIME	30	27
GENTAMICIN	10	21
SULFORAPHANE	8mcg	15

Table 1. AST TABLE

The above results indicate that sulforaphane is able to greatly inhibit the growth of *P. aeruginosa*. Moreover when compared the zone of inhibition of SFN against various antibiotics an appreciable value is observed. SFN at 8mcg concentration is showing 15mm zone of inhibition, which is comparable with the antibiotics Amikacin, Gentamicin and Norfloxacin at 10-mcg concentration. In addition, sulforaphane is a potent inhibitor of the pyocyanin pigment as there is absence of green pigment surrounding the zone of inhibition of SFN. This remarkable property of inhibiting both pigment production and biofilm formation is of great interest and further study in depth must be carried out to find out more about how the compound interacts with the pathogen *P. aeruginosa* and by what means it inhibits the QS property of *P. aeruginosa*. (Refer Table 1. and Fig 1)

V. CONCLUSION

The organism, *P. aeruginosa*, is emerging as a dreadful nosocomial pathogen, with its evolving drug resistance and the amazing adaptability to diverse environments. Hence, it is indispensable to develop new drugs, not only to control the infections, but also to counteract the resistance phenomena. It can be observed that an enormous resource of active metabolites has been contained in the huge diversity of plants on earth and only a small portion has been discovered with the quorum quenching potential. Not only is it necessary to explore more plant sources for the anti-QS activity, it is also very important to promote the known phytochemical QS inhibitors as anti-pathogenic drugs, before the pathogen evolves to defeat all the available antibiotics.

Inhibitors based on QS system have been suggested as a means to counter measure the antibiotic resistance mechanism in organisms. Thus, compounds found in natural sources like plants, vegetables etc are finding uses in current treatment procedures. In this aspect broccoli was used to extract sulforaphane and FTIR spectroscopy was carried out, which showed the presence of required functional groups. Thus based on results obtained, it can be concluded that sulforaphane is present in the powder obtained after extraction.

Thus, sulforaphane can be concluded as a potent QSI based on the results obtained and further studies can be carried out to study the effect of sulforaphane in detail. Moreover sulforaphane being derived from natural source i.e. broccoli can be easily acceptable in the form of tablet and hence sulforaphane is a potential alternative for treating *P. aeruginosa* infections in cases of antibiotic resistance.

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