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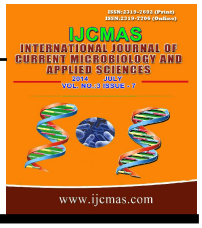

Original Research Article

Syzygium aromaticum - A common food spice with potential Quorum quenching activity on *Serratia sps YAJS*

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A B S T R A C T

Syzygium aromaticum is an aromatic flower bud often referred to as clove. It has medicinal properties and used in Indian Ayurveda and Chinese medicines. Clove is known to have antibacterial activity but there are very few reports on quorum quenching activity. Hence an attempt was made to check the efficacy of food spice *Syzygium aromaticum* for its quorum quenching activity against *Serratia sps YAJS*. This organism is known to cause nosocomial infections whose virulence character expression is controlled by quorum sensing circuits. Aqueous extracts of spice were tested for growth, virulence characters like prodigiosin, protease, Dnase and swarming nature in *Serratia* sps. The spice was found to inhibit all the virulence characters except for Dnase. Quenching activity was further confirmed on bio indicator organism *Chromobacterium violaceum 12472* in which inhibition of violacein pigment production was demonstrated. Further studies on quorum quenching ability of *Syzygium aromaticum* could be promising for curing of bacterial infections without causing selective pressure for emergence of antibiotic resistant strains.

Keywords

Syzygium aromaticum, *Serratia sps YAJS*, Quorum quenching, *Chromobacterium violaceum 12472*

Introduction

Syzygium aromaticum is an evergreen tree which belongs to the family Myrtaceae, that has numerous medicinal properties. It is often referred to as clove bud which has a shaft and a head. Clove is mostly used as spice and its oil is used essentially in Indian Ayurveda and Chinese medicine for treatment of many ailments. Major component in clove oil which imparts aroma is Eugenol, a phenyl-propanoid class of chemical compound . It contributes 72-90 % pleasant, sweet aromatic fragrances to the clove-bud. Eugenol has local anesthetic and antiseptic properties, hence useful in dental care essentials as well as in treatment procedures. Clove is also rich in minerals like phosphorus, sodium , hydrochloric acid, iron, calcium, potassium, vitamin A & C (Srivastava *et al* 2005).

The health benefits of clove oil can be attributed to its antimicrobial, antifungal, antiseptic, antiviral, aphrodisiac and stimulating properties. The oil is used for

treating a variety of health disorders including toothaches, indigestion, cough, asthma, headache, stress and blood impurities. The most important and common use of clove oil is in dental care. Several toothpastes, mouth wash and oral care medications contain clove oil as an important ingredient. These compounds are known to have antioxidant properties (Viuda- Martos, M. et al., 2010). Consumption of natural foods rich in flavonoids helps to protect the body from lung and oral cavity cancers.

Many research papers reported the antimicrobial activity of *Syzygium aromaticum* against different gram positive and gram negative bacteria but very few reports were published on Quorum sensing inhibition activity of *Syzygium aromaticum.* Quorum sensing (QS), a bacterial cell communication mechanism enables bacterial populations to cause infection. Recent research has revealed that eukaryotes are capable of interfering with bacterial communication by the production of molecular signals that interact with the bacterial QS system and attenuate the virulence of the bacterial pathogen (Quorum Sensing Inhibition). Dietary phytochemicals with QSI activity are of therapeutic interest, as their presence in the diet may positively affect the intestinal flora and prevent pathogen invasion. Continuous efforts of researchers in the quest for new therapeutics have opened up a new era where phytochemicals are being investigated as QSI agents.

Disruption of quorum sensing (Quorum Quenching) attenuates the pathogenicity in bacteria without imposing resistance. Hence wide range of naturally occurring substances, particularly plants extracts, have been evaluated for their ability to modulate quorum sensing in Gram-negative bacteria (Vattem, D. *et al* 2007). In a recent study Sandy Siew-Mian Yeo and Foong -Yee Tham (2012) screened twenty traditional Chinese medicine plants commonly used in South-East Asia for QS inhibitors using two biomonitor strains, *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1. In another paper published by Thiba Krishnan, Wai-Fong Yin and Kok-Gan Chan **(**2012) reported the anti-quorum sensing activity of an Ayurveda spice, clove (*Syzygium aromaticum)*. These authors demonstrated that clove extract inhibited quorum sensing-regulated phenotypes in *Pseudomonas aeruginosa* PA01, including expression of *lecA::lux* (by hexane extract), swarming (maximum inhibition by methanol extract), pyocyanin (maximum inhibition by hexane extract). However QSI activity of clove on *Serratia* has not been reported so far. Since clove is exclusively used in food, attempts were made to study QSI activity of aqueous extracts rather than solvent extracts of *Syzygium aromaticum* on *Serratia sps YAJS.*

Materials and Methods

Microorganisms

Serratia marcescenes: Isolate obtained from rhizosphere soil samples of college campus which was characterized by morphological, biochemical methods and by 16s rRNA analysis as *Serratia marcescenes* (16srRNA sequence was deposited in GenBank under acc.no:JQ21730). It was named as *Serratia sps YAJS* and used for QSI activity throughout the study.

Chromobacterium violaceum 12472: Bio indicator for QSI activity, was obtained from Dr. Hammeda bee, Asst.Professor, Department of Microbiology, Osmania University.

Quorum quenching activity on *Serratia* isolate

The aqueous extracts of *Syzygium aromaticum* was prepared for QSI activity analysis. The spice powder was dissolved in aqueous extracts to get a final concentration of 10mg/ml, filtered using 0.2 micrometer pore size filter and then used to test Quorum sensing inhibitory activity. Serial dilution of the crude extract of food spice was made in nutrient broth and 0.1 ml of overnight grown culture of *Serratia* was added and incubated over night at 30^{o}C.

After incubation, growth was monitored by recording absorbance at 600nm. Since the production of protease, prodigiosin were quorum sensing dependent, these parameters were monitored in the treated samples at all dilutions. Prodigiosin assay was done as described by Slater *et al*., 2003 and protease activity by Anson (1938) and Folin O and Ciocalteau(1929) method.

Swarming activity was determined by Daniel B. Kearns (2010) method. The swarm agar plates were prepared as recommended and 250 µL of spice extract was seeded with 5 mL of the agar and poured immediately on a 10 mL of pre-warmed agar plate as an overlay. Two hundred microliters of the bacterial culture was inoculated at the center of the agar surface and the plate was incubated for 16 hours at 37 °C.

Dnase activity was monitored by Jeffries *et al*, (1957) method. Enzyme production was tested on DNA agar by inoculating the culture as a thick band at the center of the plate. The plate was incubated at 32^{o}C overnight. After incubation the plate was flooded with 2N HCl. Dnase production was identified as a halo zone of clearance (DNA degradation) around the culture streak.

QSI activity on *Chromobacterium violaceum12472*

QSI activity of crude extracts of spice was tested by violacein inhibition assay using *Chromobacterium violaceum 12472* as bio indicator organism. The nutrient agar plates were pre-seeded with overnight grown culture of *Chromobacterium violaceum 12472*. Wells were made in the agar plate with a sterile cork borer of 6mm in diameter. The wells were loaded with 50µl of the sample to be tested and incubated overnight at 30^{o}C. The QSI activity was calculated by measuring the diameter of colorless haloes created due to inhibition of violacein pigment but not the growth (Maryam Zahin, 2010).

AHL extraction

5ml of overnight grown culture was used for AHL extraction. Cells were separated by centrifugation and supernatants were extracted twice with equal volumes of ethyl acetate. The extracts were evaporated to dryness. Later they were dissolved in 50-100 µl of ethyl acetate (Shaw *et al.*, 1997) and used for further studies.

NMR Analysis

Structural identification of AHLs produced were identified by NMR analysis which was carried at IICT, Hyderabad. 1H NMR spectra were recorded on Bruker-300 spectrometer (300 MHz) and Varian Unity 500 spectrometer (500 MHz) in DMSO using TMS as internal standard.

Mass spectroscopy

The AHL samples were extracted as mentioned above and subjected for MS analysis. Samples treated with and without spice extract were subjected to analysis for

comparison. Mass spectral analysis was performed at IICT, Hyderabad. Mass spectra were recorded on Finnigan MAT 1020, mass spectrometer operating at 70 eV.

Phytochemical Analysis

Aqueous crude extract of *Syzygium aromaticum* were subjected to qualitative analysis for identification of phytochemicals present and TLC by standard methods (Sunil H. Ganatra. et al., 2012).

Statistical Analysis

All experiments were performed in triplicates and standard deviation was calculated to all the samples in MS Excel. The data was also analyzed by ANOVA and one way variance using SPSS package. All the graphs were represented with error bars.

Results and Discussion

Serratia is an opportunistic pathogen, which causes a variety of infections. *Serratia marcescens* is involved in nosocomial outbreaks in intensive care units. *S. marcescens* causing nosocomial infection represents serious problem worldwide (Ivanova *et al.,* 2008; Po-An *et al.,* 2010). *S. marcescens* could cause Cucurbit yellow vine disease (CYVD) in plants such as squash, pumpkin, watermelon, rice and cotton (Zhang *et al.,* 2005). It is also known to cause food spoilage. The *Serratia sps YAJS* employed in present study was isolated and characterized as *Serratia marcescens* with 95% similarity by phylogenetic analysis (Y. Aparna and J. Sarada, 2012).

Serratia produces N Acetyl Homoserine lactone as quorum sensing signaling molecule which is used for regulation of genes for extracellular virulence factors like prodigiosin, swarming motility, secretion of enzymes like protease and Dnase. These parameters were monitored in the treated samples. The results obtained during the study with aqueous extracts of *Syzygium aromaticum* against *Serratia sps YAJS* were graphically represented.

Serratia growth was reduced to 16.5 % at 2 mg/ml concentration which has reached to 44.9% at 8mg/ml concentration (Graph 1a). These results indicate that it has antibacterial activity. Concentration dependant reduction in growth pattern was observed. Sabahat Saeed and Perween Tariq (2008), reported that aqueous decoction of *Syzygium aromaticum* did not inhibit the growth of *Serratia marcescens* by disc diffusion method. However present data indicated approximately 45% reduction in growth. This could be because of the concentrations used for testing (2-8mg/ml) and the method employed. The antibacterial activity observed in present study was due to the presence of Eugenol and its derivatives in *Syzygium aromaticum* which was known to damage bacterial membranes. (Hemaiswarya, S and Doble, M., 2009).

Interestingly, spice had quorum quenching ability as it effectively reduced the QS regulated virulent factors like prodigiosin, protease activity, and swarming nature. Relative prodigiosin values were observed to be influenced as there was decline in values from 43.27% at 2mg/ml concentration to 90.3% at 8mg/ml respectively (Graph 1b). Graph 1c reveals that there was reduction in protease activity also. With the increase in the concentration of the spice extract there was simultaneous reduction and maximum 99.28% at 8mg/ml concentration was recorded.

QS-regulated swarming motility has been characterized as form of flagella-dependent

movement on viscous environments such as semi-solid agar surfaces. Swarming nature of *Serratia* was also found effected with the treatment as there was 60% reduction (Fig 1) at 8mg/ml concentration. From these results it was evident that the phytochemicals of spice aqueous extract were effective in reducing the three virulent factors of *Serratia* tested. However, Dnase activity of *Serratia* was not affected by the treatment. Apart from the antibacterial activity of *Syzygium aromaticum* , the aqueous crude extract showed high QSI activity by effecting virulence factors like prodigiosin production by 90.3% reduction, 99.3 % reduction in protease activity and 60% reduction in swarming nature of *Serratia*. All these factors were reported to be controlled by AHL quorum sensing regulatory mechanism in *Serratia sps YAJS.*

QSI activity on *Chromobacterium violaceum*

As most of the research papers to date on quorum quenching activity of plant extracts were tested against the bio-indicator organism *Chromobacterium violaceum,* present study was also extended to test spice extract on *Chromobacterium violaceum 12472*. This was performed by studying violacein inhibition assay, a plate based method.

Crude aqueous extract was loaded on to Luria Bertani (LB) plates spread with *C. violaceum* and incubated overnight at 30°C. QS Inhibition was detected by a ring of colorless, but viable cells around the well. The halo-effect is created by pigment less (QS interrupted) cells adjacent to the wells. Loss of purple pigment in *C. violaceum* is indicative of QS inhibition by spice aqueous extract. Strong anti-QS activity was observed with aqueous extracts against distilled water as negative control .The halos produced on lawns of the biomonitor strain could be the result of either (i) inhibition of cell growth or (ii) quenching of QS signals. Growth inhibition would produce a clear halo versus a turbid halo for the latter (Adonizio, 2008). To differentiate, the halo was examined under a higher magnification. Clear halos against violet background around the sample wells were measured (Fig 2).

Interesting observations were made in this assay as antibacterial activity was found associated with quorum quenching activity. Antibacterial activity of *Syzygium aromaticum* extract was observed at 6mg/ml. However quorum quenching activity i.e. violacein inhibition activity was observed even at low levels (2mg/ml) of concentrations tested (Graph 2). These results indicate the presence of strong quorum quenching compounds in the aqueous crude extract of *Syzygium aromaticum.*

The type of signaling molecule produced by the *Serratia* isolate was analyzed by extracting AHL molecules from the culture supernatants. The extracted samples of AHL's were analyzed by NMR and identified as C6HSL (Fig 3).

To know the fate of AHLs for hypothesizing probable mechanism of quenching activity the treated samples were subjected to Mass spectroscopy. MS analysis revealed the cleavage of C6AHL at the acyl side chain which was evident from the fragmented peaks observed in MS analysis report at m/z 102 (Figure 4). A peak at m/z 102 indicates the characteristic Homoserine lactone ring alone and the fragmented acyl side chain were indicated by small peaks. Dilara *et al,* 2008 reported the fragmentation of AHL molecule by Mass spectroscopy which stands as support to the present data

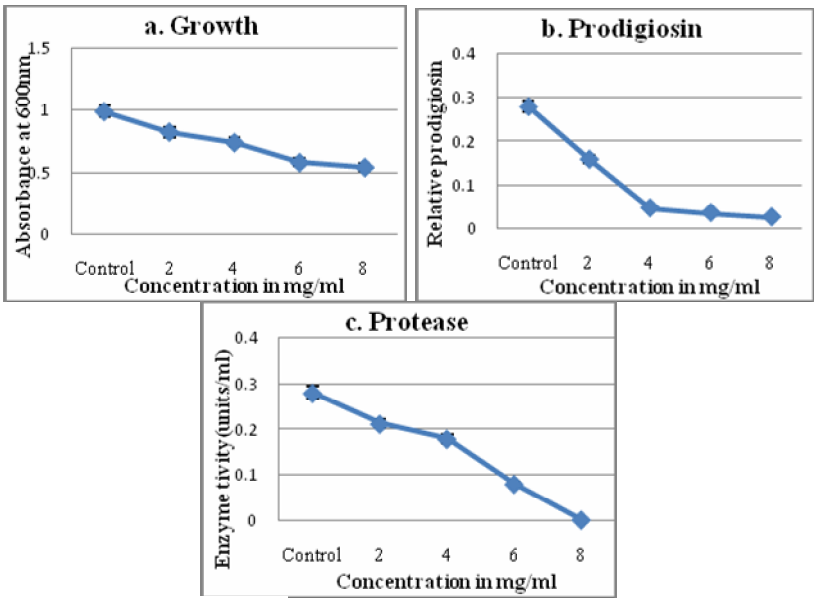
obtained. MS analysis reveals the fact that AHL molecule is subjected to lysis or degradation. The inactivation of AHL could be by cleaving the molecule or separating their lactone ring from the side chain.

The phytochemicals responsible for QSI activity present in the extract were analyzed. As most of the plant extracts are known to possess a mixture of phytochemicals like phenols, tannins, flavonoids, quinones, saponins, cardiac glycosides, terpenoids, sterols, alkaloids, resins, coumarins, volatile oils, phlobatannins, anthraquinones, peroxides etc., specific qualitative tests were performed for the detection of these phytochemicals. Based on qualitative reactions performed, the following phytochemicals i.e Quinones, saponins, terpenoids, and tannins were identified in aqueous extract. (Fig 5).

Efforts were made to separate these phytochemicals in the crude extract by TLC analysis wherein silica gel was stationary phase and Ethylacetate : Methanol : water (3:0.5:0.5) was mobile phase (Fig 6). Two spots of saponins, terpenoids were observed when TLC sheet exposed to Iodine fumes. Further identification and characterization of these phytochemicals were being carried out by fractionation of crude extract using different solvents.

This study shows that the presence of phytochemicals that exhibit anti-quorum sensing activity in the clove aqueous extracts may be useful as the quorum quenching agents. Further studies on QSI ability of *Syzygium aromaticum* can be promising for curing of bacterial infections without causing selective pressure for emergence of antibiotic resistant strains.

Graph.1 Effect of *Syzygium aromaticum* on virulence factors of *Serratia sps YAJS*



a. Growth was recorded as A600 nm
b. Relative prodigiosin expressed as D600/OD540nm.
c. Enzyme activity expressed as units/ml.
**Error bars indicate standard deviation

Graph.2 Comparision of quorum quenching activity and antibacterial activity of *Syzygium aromaticum* extract

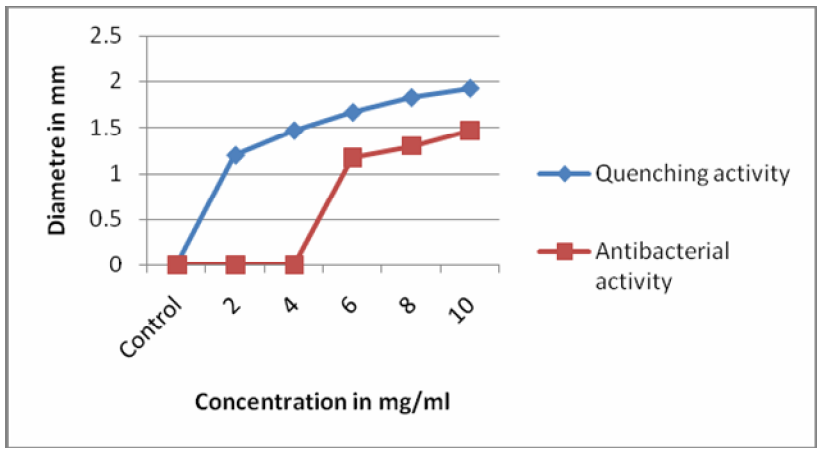
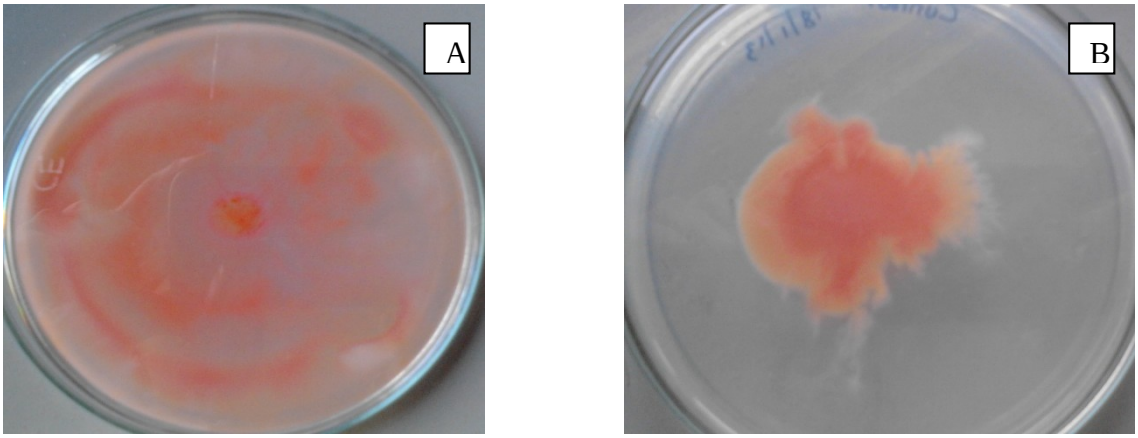


Fig.1 Inhibition of swarming nature by *Syzygium aromaticum* extract



Reduction in swarming nature of *Serratia sp YAJS.*
A-Control B-8mg/ml concentration

Fig 2: QSI activity of *Syzygium aromaticum* extract on *Chromobacterium violaceum12472*

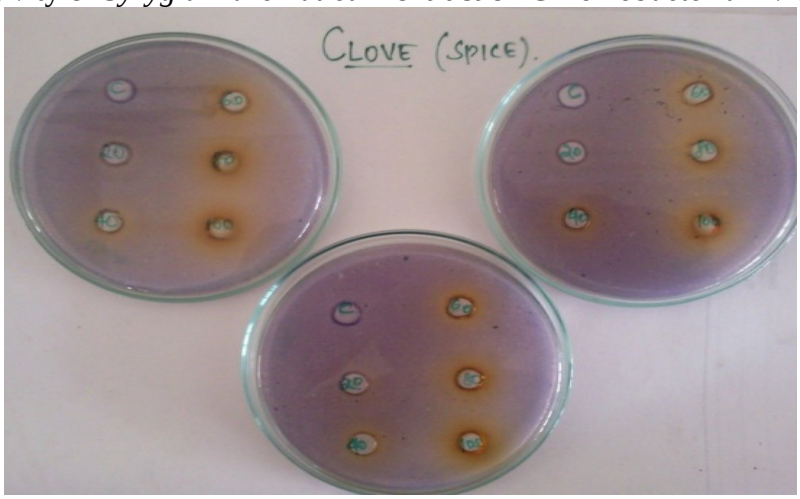


Fig.3 NMR spectral analysis of AHL produced by *Serratia sps YAJS*

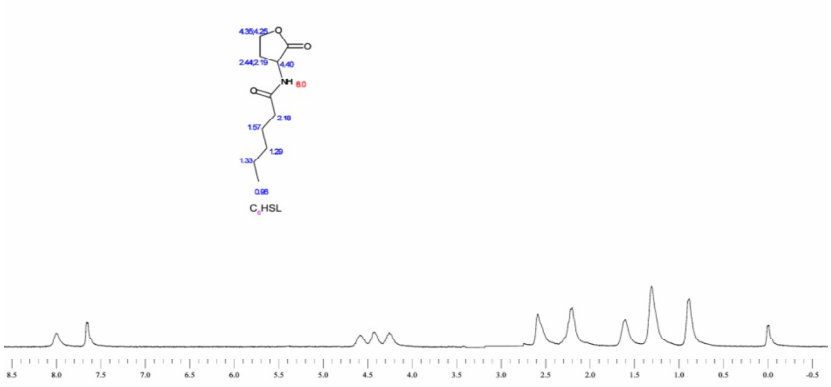


Fig.4 Mass Spectral analysis of AHL in *Syzygium aromaticum* (8mgl/ml) treated sample

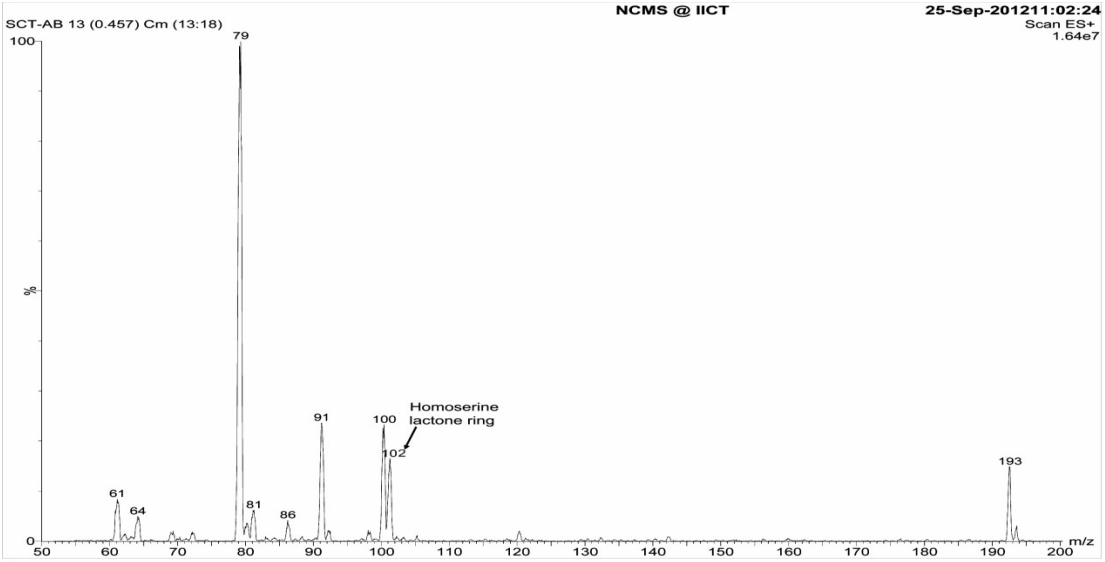


Fig.5 Phytochemical analysis of clove extracts

	Phenols	Flavonoids	Quinones	Tannins	Saponins	Cardiac Glycosides	Steroids	Terpenoids
Aqueous	+/-	-	+	+	+	+/-	+/-	+
Ethanol	+	+	+	+	-	+/-	-	+
Methanol	+	+	+	+	-	+	-	+
n-hexane	-	-	+	-	-	+	+	-
Acetone	-	+	+	+	-	+	+	-
Chloroform	-	+	+	-	-	+	+	+

Fig.6 TLC of aqueous clove extract

Methanol:water:Formic acid(18:9:1)

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