Advanced Column Chromatography Techniques for Comprehensive Extraction, Isolation and Characterization of Allicin in Garlic Extracts and their antibacterial activity

Mayur S. Tekade[1*], Pallavi M. Patil[2]

[1,2]Department of Pharmaceutical Chemistry, PES's Modern College of Pharmacy, Yamunangar, Nigdi, Pune, Maharashtra, India & affiliated to Savitriai Phule Pune University, Ganeshkhind, Pune, Maharashtra, India

Corresponding Author- Mayur Sanjay Tekade

Department of Pharmaceutical Chemistry, PES's Modern College of Pharmacy, Yamunangar, Nigdi, Pune, Maharashtra, India & affiliated to Savitriai Phule Pune University, Ganeshkhind, Pune, Maharashtra, India

Abstract:

This Study is to investigate anti-bacterial study of allicin by isolating the allicin from Allium Sativum by using the column chromatography method. The extraction, purification, isolation, and identification of the Allicin were achieved by using Column, IR, and Mass and NMR spectrometry. We compared our Isolated Allicin result to the marketed Allicin we found that our values are closer to marketed Allicin. Compared Isolated Allicin with Marketed Allicin, the Isolated Allicin are comparatively similar upto 92-95% to the Marketed Allicin. In future we can isolate and characterised a number of other constituents from Allium sativum and the proposed analytical methods will be useful for estimating these drug. Ultimately, our investigation showed that garlic extract had antibacterial properties against MRSA in vitro. Additionally, our results indicated that garlic extract and cefoxitin had synergistic effects.

Keywords: Column Chromatography, IR, Mass, NMR Spectrometry, Allicin, Antibacterial Study

1. Introduction

Allium sativum L., commonly known as garlic, is a widely used culinary ingredient and condiment in many indigenous cultures.[1] Enzymatic cleavage of alliin, which yields the pungent chemical allicin, is one of its abundant sources of organo-sulfur compounds.[2-3] Allicin has a distinctively strong flavor, but it may also have positive effects on human health, such as antibacterial, anticancer, and cardiovascular health advantages and cholesterol reduction.[4-5] Garlic's allicin has been studied for its culinary and medicinal qualities, which has led to research on its extraction and measurement.[6]

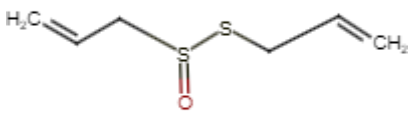

Fig. 1 Structure of Allicin

Most of the physiological and therapeutic properties of garlic are attributed to the thiosulfinate functional group of allicin. This chemical, which is not a naturally occurring byproduct of the plant, is produced at the expense of alliin, a form of cysteine sulfide.[7] As the tissues of garlic plants break down, the most important enzyme in this family of plants, alliinase, mixes with alliin to produce pyruvic acid and the very unstable chemical allicin.[8-9] The amount of this composition is determined by external elements and processing conditions, however these parameters can be changed to prevent the loss of this valuable material.[10] Allicin and thiosulfinates absorb free radicals and also stimulate fibrinolysis, inhibit platelet aggregation and lipid peroxidation, and decrease blood lipid levels[8,10] Industries are increasingly recognizing the importance of non-toxic and reliable extraction methods due to the rising demand for natural ingredients and eco-friendly alternatives that avoid harmful substances.[9,11] This shift has led to a greater focus on developing commercial extraction techniques that minimize or eliminate the use of chemical solvents. These innovations aim to extract valuable compounds from plant materials more efficiently, resulting in purer products and broader applications across various fields.[11]

Allicin is listed as an active component having many targets by the World Health Organization. DNA synthesis is also inhibited by allicin.[12] It is an electrophile that inhibits protein synthesis by interacting with glutathione, coenzyme A, and cysteine.Two Garlic oil not only has antibacterial properties but also shows antifungal properties against Candida albicans.[13] Garlic has antihistaminic properties and reduces allergic reactions in the airways, according to Kyo et al.One Methicillin-resistant Staphylococcus aureus (MRSA) and multidrug-resistant (MDR) types of bacteria are common throughout the world, making the treatment of infectious infections difficult.[14] A gram-positive bacterium is called S. aureus.

Infections brought on by S. aureus that are acquired in the community typically resolve on their own with appropriate antibiotic treatment.[15] On the other hand, S. aureus obtained in a hospital is one of the most prevalent microorganisms that cause diseases linked to healthcare. MRSA strains emerged as a result of S. aureus developing more antibiotic resistance.[16] MRSA infections raise the morbidity and death rates of infected patients and are serious, sometimes fatal clinical issues.[17] MecA is in charge of these strains' drug resistance.[18] Here, our goal was to ascertain whether garlic extract could combat MRSA

medication resistance. We also assessed the MRSA strains' in vitro susceptibility to the garlic extract.

2. Experimental

2.1. Instrumentation

2.1.1. Column chromatography

Cylindrical chromatographic column, (Height of column: 40 cm; Diameter of column: 3.5 cm), Separating funnel, Cotton, Iodine chamber, TLC plates, TLC plate developing chamber, Capillary tubes

2.1.2. Mass spectrometry

A triple quadruple mass spectrometer, the Shimadzu HPLC- MS/MS type API 3000, was used for the turbo spray and MS–MS experiments. By varying the capillary voltage between +5000 and 4500 V, positive and negative electrospray MS data were acquired. The collision energy was ramped from 30 to 60 V in the nitrogen atmosphere to create the MS–MS data.

2.1.3. NMR spectroscopy

The 1H NMR were recorded on Varian Mercury plus 400 MHz, using DMSOd6, solvents and trimethylsilane (TMS) as the internal standard.

2.1.4. FT-IR spectroscopy

The IR spectra were acquired using a Perkin Elmer 1600 series FT-IR spectrophotometer in the solid state as KBr dispersion medium.

2.2. Material and reagents

Pure standard Allicin sample was purchased by Yucca Enterprises Wadala East Mumbai (India) with a purity of 97.58% based on the company certificated. Analytical grade solvents were used. Organic solvents, Silica gel (60-120#), n-Hexane (need for packing of column)

2.3. Method

2.3.1. Column Chromatography of Active Extract of Allium sativum

Extraction:

***Collection of plant materials*:**

Allium sativum from different cultivation site were collected i.e Garlic Extract (G1, G2, G3, G4) and brought to the laboratory for further analysis.

Processing of plant materials:

The collected A.sativum bulb from different cultivation sites were cleaned thoroughly and dried under shade. The dried bulb was blended into fine powder and stored in air tight container at room temperature.

Preparation of extracts:

The organic solvents such as petroleum ether and distilled water was used for the extracting the bioactive compounds from bulb of A.sativum. The extraction was done using soxhlet apparatus and hydrodistillation was carried out in Clevenger apparatus. The extract dried using vacuum evaporator and stored in air tight containers.

Isolation and Characterisation:

In preliminary phytochemical test of rhizomes of *Allium sativum* petroleum ether and water extract showed presence of allicin. Also the TLC of both extract of shows presence of different phytometabolites. So for the further synthesis allicin need to be isolate using column chromatography. About 10 gm. of extract was subjected to column chromatography to obtain further separated fractions.

Slurry of adsorbent (the activated silica for column) was prepared by mixing it with the solvent (mobile phase) and poured the mixture into the glass tube which contained the column solvent. The cotton served to give a flat base to the column of adsorbent, which was placed into the tube before pouring the slurry. Adsorbent was allowed to settle after some time sample was loaded. The test sample was prepared by mixing the extract with silica powder until it became free flowing.

Table1. Experimental column chromatography

Parameter	Petroleum ether extract	Water extract
Height of column	40 cm.	40 cm.
Diameter of column	3.5 cm	3.5 cm
Stationary phase	Silica gel for column chromatography (60-120#)	Silica gel for column chromatography (60-120#)
Mobile phase	1.n-Hexane, 2.Ethyl-acetate 3. Methanol	1.Petroleum-Ether 2.Ethyl-acetate 3. Methanol
Flow rate	6-8 drops per minute	6-8 drops per minute
Number of fractions Collected	5	5
Volume of each fraction	200 ml	200 ml

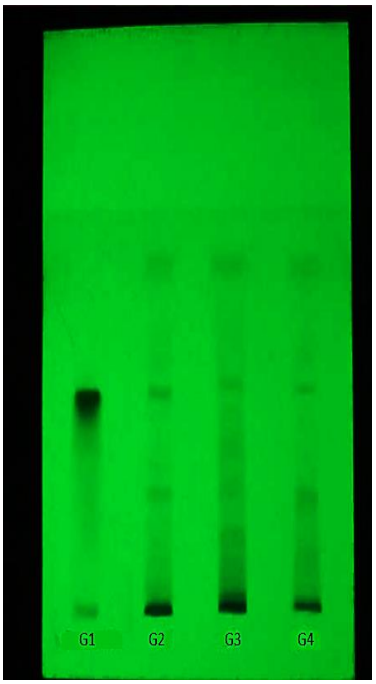

Fig.2 Petroleum Ether Extract of Garlic

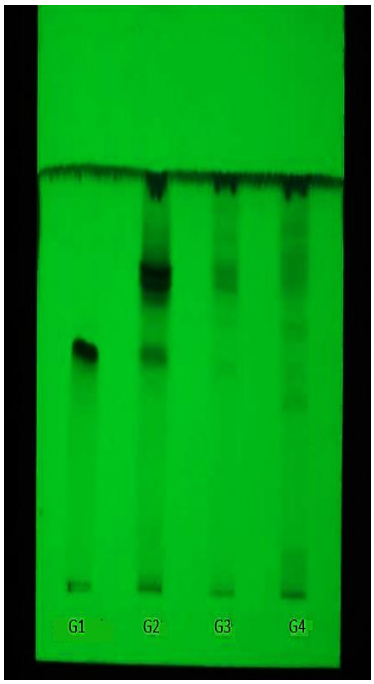

Fig.3 Water Extract of Garlic

Cotton was placed above the sample loaded to avoid disturbances to sample as fresh mobile phase was added to the column. During fractionation of column the level of the solvent was never allowed to fall below the level of the test sample loaded. Then the column was eluted with mobile phase in isocratic manner. Fractions of desired volume were collected and dried at room temperature to obtain a more concentrated fraction**.**

2.4. Bacterial isolation

Clinical specimens (pus) were gathered and cultivated on mannitol salt agar supplemented with nutrients. With the appropriate controls, a number of biochemical tests were carried out, including as Christen's urease assays, mannitol motility, catalase, coagulase, and gram staining.[19] Additionally, penicillin, gentamycin, cotrimoxazole, clindamycin, erythromycin, ciprofloxacin, vancomycin, and linezolid were used in antimicrobial susceptibility tests. For the MRSA screening, cefoxitin discs were utilized.

Bactericidal activity

We measured the minimum inhibitory concentration (MIC) to evaluate the bactericidal activity. Garlic extracts in aqueous form and the culture were ready. We employed a range of dilutions of aqueous garlic extract (16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µL). A 24 hour incubation period at 37°C was then observed after adding 1 µL of the MRSA inoculum. Next, the nutrient agar plate was divided into spots for each dilution, and the microliter plate was kept at 37 °C for 24 hours[20].

Applying the agar well diffusion method, garlic's antibacterial properties on S. aureus were evaluated.

A nutritional broth was filled with MRSA inoculum, which was then left to incubate overnight. Next, a syringe puncture was used to create a cylindrical bore after the culture was swabbed onto the Mueller-Hinton agar plate. The zone of inhibition was detected when the aqueous garlic extract, positive and negative controls, and a micropipette (10, 25, 50, and 100 µL) were pipetted into the cylindrical bore and incubated for 24 hours at 37 °C. A zone of inhibition is a sign that the medication is working.15 Garlic's antibacterial properties are evaluated using the agar well diffusion method, as seen in Figure 4.

Fig.4.The anti-MRSA bactericidal activity of allicin

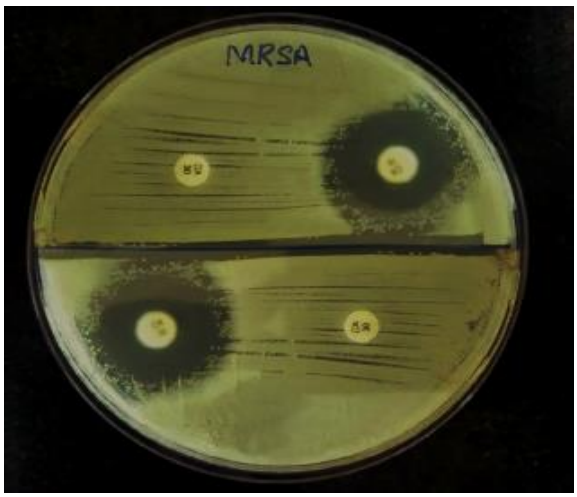

Fig.5.Augmented activity of Allium sativum with commercial Cefoxitin disc

Garlic extract is infused into the Marketed cefoxitin disc.

Using Marketed cefoxitin discs, we evaluated A. sativum's activity in addition to determining its susceptibility to MRSA because of this. On the Mueller-Hinton agar plate[21], an MRSA inoculum was swabbed.Eighteen Following that, a cefoxitin disc containing 50 µL of garlic extract was added to the Marketed cefoxitin that had been placed on the Muller-Hinton agar plate. After 24 hours at 37°C, the plate was incubated.

Table2. Concentration vs. Zone of Inhibition

Sr. No	Concentration (µL)	Zone of Inhibition (mm)
1.	100	20
2.	50	16
3.	25	10
4.	10	5

3. Result and Discussion

3.1. Structure Elucidation of Allicin

The IR spectrum (KBr) was characterized by the absorption frequency of C-H (strch) 2974.66 cm-1 and C-S (strch) 926.62 cm- 1, C=C (strch) 1389.83 cm-1, and S=O at 863.95cm-1and S-S 745.352cm-1. The mass spectrum was characterized by the appearance of the molecular ion peaks at 163.02 (M & M+2) which confirm the molecular weight of the suggested Allicin in Table 3. The 1H NMR spectrum (DMSO-d6) showed an signal at δ 3.86 (2H, d, J = 7.0 Hz), 4.93 (2H, d, J = 8.9 Hz), 5.00 (dd, J = 10.7, 2.2 Hz), 5.47 (dd, J = 16.7, 2.2 Hz), 5.82 (dd, J = 10.8, 2.2 Hz)), 5.83 (1H, ddt, J = 16.7, 10.8, 8.9 Hz), 6.04 (1H, ddt, J = 16.5, 10.7, 7.0 Hz).

In this case, out of the 200 exudate samples that were obtained, 150 displayed gram-positive cocci that were evenly stained and grouped in pairs. In addition, 118 samples developed artificial non-selective culture conditions into colonies with pigments that were golden yellow in color. Results for MRSA were positive in 52 out of the 118 samples. Once isolated, one was chosen and prepared for biochemical reactions. A mannitol fermentation and urea hydrolysis occurred during the testing. When the coagulase test was positive, the catalase test resulted in effervescence. Following the Central Laboratory Standard Institute 2022 standards, the isolate was classified as MRSA since it exhibited cefoxitin resistance.
Remarkably, even at the lowest dilution (1 µL), the aqueous garlic extract demonstrated potent antibacterial action. Garlic's organosulfur components, which prevent microbial development, may be the cause of its bactericidal action. At the highest concentration, there was a greater zone of inhibition for the agar dilution. The extract concentrations in the 20 mm with 100µL, 16 mm with 50 µL, 10 mm with 25 µL, and 5 mm with 10 µL zones of inhibition in the agar well diffusion tests were as follows shows in Table 1. Furthermore, there was evidence of synergistic activity between the cefoxitin disc and the garlic extract. In contrast to the cefoxitin disc alone, which did not exhibit any zone of inhibition, this combination created a 24-mm zone of inhibition. Fig. 6 demonstrates the marketable cefoxitin disc's action against A. sativum.

Many people utilize garlic as a herbal remedy. Garlic's antimicrobial properties have been documented in numerous investigations. Garlic has no antibacterial properties at doses below the minimum inhibitory concentration (MIC), according to Daka et al.[22] The variation among the garlic species or strains could be the cause of this. Furthermore, Durairaj et al. found that an aqueous garlic extract at 100% concentration produced an inhibitory zone of 33 nm.[23] Grampositive Bacillus spp. are more susceptible to garlic than other species, according to research by Durairaj et al. Our findings agree with those of Atheer, who found the greatest known concentration of garlic—100 mg/mL—to have a zone of inhibition of 25 nm. Apart from its impact on MRSA, 10% methanol garlic extract (MBC: 1 mg/mL) was found to have antibacterial properties against Vibrio alginolyticus and V. harveyi by Natasya-Ain et al.[24] Garlic extract has been demonstrated in numerous investigations to exhibit antibacterial

properties against a variety of pathogens, including Salmonella typhi, Pseudomonas aeruginosa, Proteus mirabilis, and Escherichia coli. Using synergy experiments, Cirković et al. demonstrated the ethanol garlic extract's synergistic effects with other medications.[25]

3.2. Characterisation

We compared our Isolated Allicin result to the marketed Allicin we found that our values are closer to marketed Allicin. The IR spectrum (KBr) of marketed Allicin was characterized by the absorption frequency of C-H (strch) 2934.57 cm-1 and C-S (strch) 956.22 cm- 1, C=C (strch) 1409.83 cm-1, and S=O at 893.55cm-1and S-S 757.32cm-1. The mass spectrum of Marketed Allicin was characterized by the appearance of the molecular ion peaks at 162.26 m/z which confirm the molecular weight of the marketed allicin. The 1H NMR spectrum (DMSO-d6) showed an signal at δ 3.26 (2H, d, J = 7.0 Hz), 4.33 (2H, d, J = 8.9 Hz), 5.00 (dd, J = 10.7, 2.2 Hz), 5.01 (dd, J = 16.7, 2.2 Hz), 5.24 (dd, J = 10.8, 2.2 Hz)), 5.63 (1H, ddt, J = 16.7, 10.8, 8.9 Hz), 6.14 (1H, ddt, J = 16.5, 10.7, 7.0 Hz).

Table3**.** FT-IR, MS and NMR of Isolated Allicin and Marketed Allicin

Sr. No.	Compound	IR	MS	NMR
1.	Isolated Allicin	C-H (strch) 2974.66 cm-1 and C-S (strch) 926.62 cm- 1, C=C (strch) 1389.83 cm-1, and S=O at 863.95cm-1and S-S 745.352cm-1	m/z Measured EI-MS Molecular ion peaks at 163.02 (M & M+2)	δ 3.86 (2H, d, J = 7.0 Hz), 4.93 (2H, d, J = 8.9 Hz), 5.00 (dd, J = 10.7, 2.2 Hz), 5.47 (dd, J = 16.7, 2.2 Hz), 5.82 (dd, J = 10.8, 2.2 Hz)), 5.83 (1H, ddt, J = 16.7, 10.8, 8.9 Hz), 6.04 (1H, ddt, J = 16.5, 10.7, 7.0 Hz).
2.	Marketed Allicin	C-H (strch) 2934.57 cm-1 and C-S (strch) 956.22 cm- 1, C=C (strch) 1409.83 cm-1, and S=O at 893.55cm-1and S-S 757.32cm-1.	Molecular ion peaks at 162.26 m/z	The 1H NMR spectrum (DMSO-d6) showed an signal at δ 3.26 (2H, d, J = 7.0 Hz), 4.33 (2H, d, J = 8.9 Hz), 5.00 (dd, J =

				10.7, 2.2 Hz), 5.01 (dd, J = 16.7, 2.2 Hz), 5.24 (dd, J = 10.8, 2.2 Hz)), 5.63 (1H, ddt, J = 16.7, 10.8, 8.9 Hz), 6.14 (1H, ddt, J = 16.5, 10.7, 7.0 Hz).

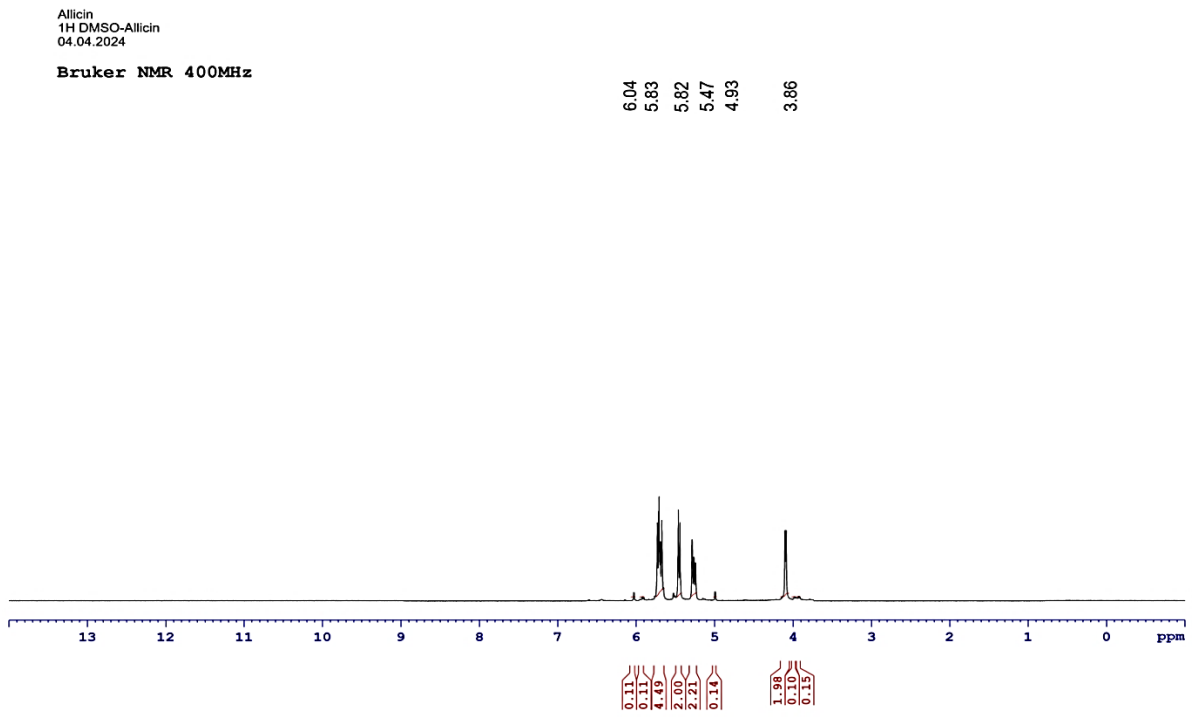

Fig.6. NMR Spectra of Isolated Allicin

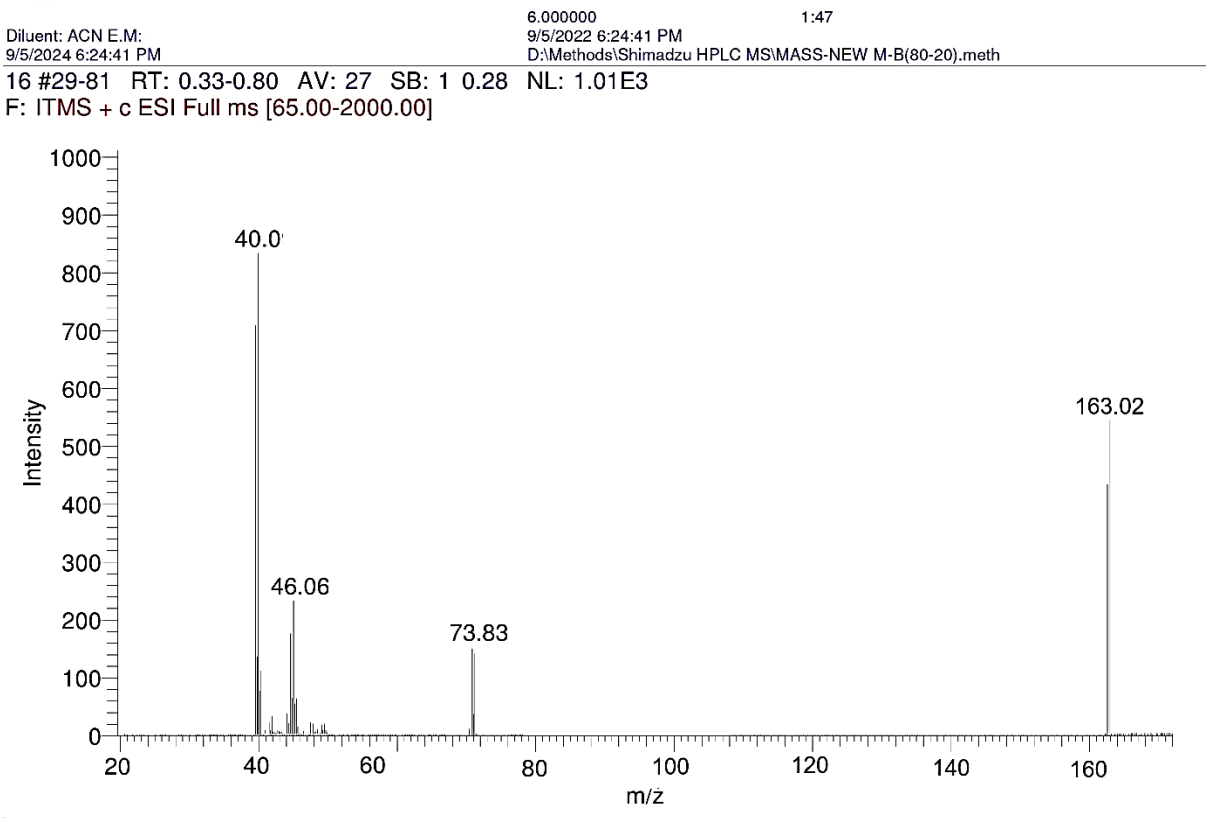

Fig7. Mass spectra of Isolated Allicin

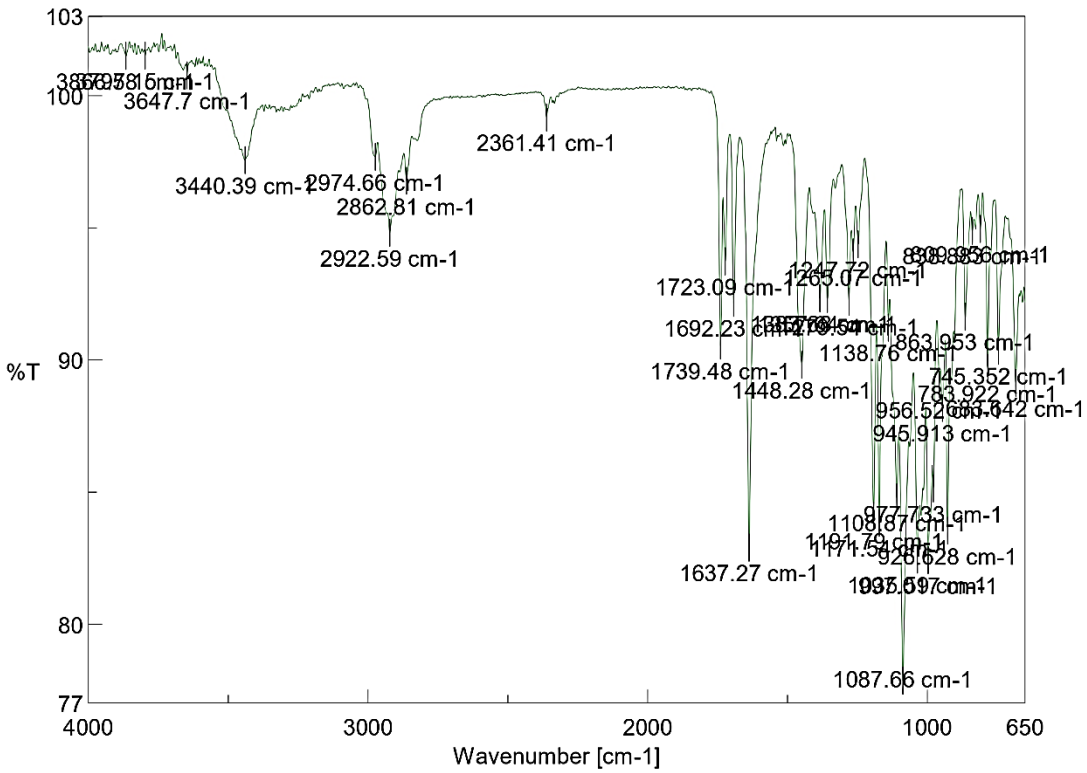

Fig8. FTIR spectra of Isolated Allicin

4. Conclusion

Column Chromatography (analytical and preparative), IR, mass spectrometry, as well as NMR (1H NMR) techniques were utilized for the determination of the process related Allicin drug substance. The extraction, purification, isolation, and identification of the Allicin were achieved by using Column, IR, and mass and NMR spectrometry. Compared Isolated Allicin with Marketed Allicin, the Isolated Allicin are comparatively similar upto 92-95% to the Marketed Allicin. In future we can isolate and characterised a number of other constituents from Allium sativum and the proposed analytical methods will be useful for estimating these drug. Ultimately, our investigation showed that garlic extract had antibacterial properties against MRSA in vitro. Additionally, our results indicated that garlic extract and cefoxitin had synergistic effects.

Financial Support: None.

Ethical Issue: Does not require an ethical approval.

Conflict of Interest: Authors declare no conflict of interest.

Acknowledgement:
I would like to express my special thanks of gratitude to my principle Sir PES'S Modern college of pharmacy Nigdi, Pune. I came to know about so many new things I am really thankful to them.

References:

1. Martins N, Petropoulos S, Ferreira IC. Chemical composition and bioactive compounds of garlic (Allium sativum L.) as affected by pre- and post-harvest conditions: a review. Food Chem. 2016; 211: 41–50.
2. Arthur S, Ewald S. Chemical investigations on alliin, the specific principle of garlic. In: Nord, F.F. (Ed.), Advances in Enzymology and Related Areas of Molecular Biology. John Wiley & Sons Publisher. 2006.
3. Randle WM, Lancaster JE. Sulphur compounds in alliums in relation to flavour quality. In: Rabinowitch, H.D., Currah, L. (Eds.), Allium Crop Science: Recent Advances. 2002; 329–356.
4. Fujisawa H, Watanabe K, Suma K, Origuchi K, Matsufuji H, Seki T, Ariga T. Antibacterial potential of garlic-derived allicin and its cancellation by sulfhydryl compounds. Biosci. Biotechnol. Biochem. 2009; 73:(9): 1948–1955.
5. Suong NTN, Desmond BW, Lynne C, Richard JH. Does garlic reduce risk of colorectal cancer? A systematic review. J. Nutr. Dis. 2007; 137: 2264–2269.
6. Rahman K, Lowe G, 2006. Garlic and cardiovascular disease: a critical review. J. Nutr. 2006; 136 (3): 736S–740S.

7. Szychowski K, Rybczynska-Tkaczyk K, Tobiasz J, Yelnytska- SV, Pomianek T, Gminski J. Biological and anticancer properties of Inonotus obliquus extracts. Process Biochemistry. 2018;73: 180-187.
8. Mozaffari NA, Shabani, S, Bayat M, Hosseini SE. Antibacterial Effect of Garlic Aqueous Extract on Staphylococcus aureus in Hamburger. Jundishapur J Microbiol. 2014; 7(11): 131-134.
9. Loghmanifar S, Roozbeh NL, Nouri HR, Jafarian S. Evaluation of nutritional value of garlic and its application in health. 4th International Congress on engineering, technology & applied sciences,New Zealand-Auckland. 2019
10. Wang Y, Jia J, Shao J, Shu X, Ren X, Wu B, Yan Z. Preservative effects of allicin microcapsules on daily foods. LWT -Food Science and Technology. 2018; 98: 225-230. doi: 10.1016/j.lwt.2018.08.043.
11. Vinatoru, M. "An overview of the ultrasonically assisted extraction of bioactive principles from herbs". Ultrasonics Sonochemistry.2001; 8: 303−313.
12. Atheer K. Antibacterial activity of garlic extract (ALLIUM SATIVUM) against Staphylococcus aureus in vitro. Journal of Bio-Science. 2014; 3: 346-348.
13. W Tesfaye, H Kuma, D Kassahun, A Beza. Anti-bacterial activity of garlic extract against human pathogenic bacteria. Journal of Pharmacovigilance. 2018; (6): 253.
14. Y Hisashi, F Kawai, E Obayashi, et al. Crystal Structures of Penicillin-Binding Protein 3 (PBP3) from MethicillinResistant Staphylococcus aureus in the Apo and Cefotaxime-Bound Forms. Journal of Molecular Biology, 2012; 423 (3): 351–64. doi:10.1016/j.jmb.2012.07.012
15. Daka D. Antibacterial effect of garlic (Allium sativum) on Staphyloccus aureus: An in vitro study. Afr J Biotechnol. 2011; 10(4): 666-669.
16. D u r a i r a j S , S r i n i v a s a n S , L a k s h m a n a P. In vitro Antibacterial activity and stability of garlic extract at different pH and temperature. Electronic Journal of Biology. 2010; 5(1): 5-10.
17. Khashan A. Antibacterial activity of garlic extract (Allium sativum) against Staphylococcus aureus invitro. Journal of Bio-Science. 2014; 3: 346-348.
18. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16: 111-120. doi: 10.1007/BF01731581
19. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGAX: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35(6): 1547-1549. doi: 10.1093/molbev/msy096
20. Natasya-AR, Eirna-LN, Jasmin MY, Karim M. Journal of Environmental Biology, 2018; 39: 808-812 doi: 10.22438/jeb/39/5(SI)/25
21. Mohamed SB, Adlan TA, Khalafalla NA, et al. Proteomics and Docking Study Targeting Penicillin-Binding Protein and Penicillin-Binding Protein2a of MethicillinResistant Staphylococcus aureus StrainSO-1977 Isolated from Sudan. Evol Bioinform Online. 2019; 15: 1- 13. doi: 10.1177/1176934319864945
22. Morris GM, Goodsell DS, Halliday RS, et al. Automated Docking Using a Lamarckian Genetic Algorithm and an Empirical Binding Free Energy Function. J

Comput Chem. 1998; 19(14): 1639-1662. doi: 10.1002/(SICI)1096-987X(19981115)19:143.0.CO;2-B

23. Morris GM, Ruth H, William L, et al. AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. Journal of Computational Chemistry, 30(16): 2785–91. doi: 10.1002/jcc.21256
24. Reiter J, Hübbers AM, Albrecht F, Leichert LIO, Slusarenko AJ. Allicin, a natural antimicrobial defence substance from garlic, inhibits DNA gyrase activity in bacteria. International Journal of Medical Microbiology; 2020; 310(1): 151359. doi: 10.1016/j.ijmm.2019.151359
25. Koneman EW, Allen SD, Janda WM, Schreckenberger PC. Color Atlas and textbook of diagnostic microbiology. 1992; 624-704.