

ANTIMICROBIAL ACTIVITIES OF SYNTHESIZED ALLICIN AGAINST DIFFERENT BACTERIA AND FUNGUS UNDER DIFFERENT PARAMETERS (PART -2)

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ABSTRACT

The pharmaceutical importance of *Allium Sativum* is due to the presence of essential metal, carbohydrate, protein, vitamins and organosulphur compounds. Allicin is one of the active ingredient of freshly crushed garlic homogenates and showed a wide spectrum activity. The antimicrobial activity of different concentration of allicin against gram positive and gram negative bacterial isolate was studied by standard protocols. The maximum zone of inhibition has been observed in *Bacillus subtilis* and minimum for *A. Hydrophila*. The minimum inhibitory concentration (MIC) varies bacteria to bacteria and

fungus to fungus respectively. The effect of temperature and pH were also studied. The antibacterial efficacy was maintained at room temperature for maximum one week and maintains for longer time only at low temperature. Antimicrobial activity of garlic come from allicin, is mainly due to S-S and S-O bond which has the ability to react with thiol containing enzyme to form S-thiolation product, the broad spectrum antimicrobial effects of allicin is due to the multiple inhibitory effects on various thiol dependent enzymatic systems. The results indicate that allicin has antimicrobial activity.

KEYWORDS: *Allium sativum*, antimicrobial activity, Richard's liquid medium.

INTRODUCTION

Despite of all progress in synthetic chemistry and biotechnology, plants are still on indispensable source of medicinal preparation in both preventive and curative. Hundreds of species are recognized as having medicinal value and many of those are considered to play a

beneficial role in health care. In recent years renewed interest has been shown in the use of medicinal plants and scientific studies are beginning to explain some of the curative phenomena associated with traditional herbal remedies. The therapeutic action of the plant depends on its chemical constituents. The botanical relationship of a particular plant to well known drug plants may be an indication of a potential therapeutic interest. Indeed, chemical relationship based on secondary metabolites specially found in certain genera and families has been observed and is made use of in botanical taxonomy. In industrialized countries health providers have reduced their dependence on the plant kingdom. The majority of developing countries still rely on herbal remedies. Indeed, phyto-medicines are beginning to link traditional (homeopathic) medicine and modern (allopathic) medicine. WHO estimates that approximately 80 percentage of the developing world's population meet their primary health care needs through traditional medicine. In India, garlic has been used to prevent wound infection and food spoilage,^[1] and as antiseptic, antiplatelet,^[2] anticancer,^[3-7] antiatherosclerotic,^[8-9] antifungal,^[10-11] antibacterial,^[12-14] antiviral,^[15] antihypertensive,^[16] antithrombotic,^[17] antioxidant.^[18-20] Garlic exhibit a broad antibiotic activity against both gram positive and gram negative bacteria that have become resistant to antibiotics^[21] and effective against many common pathogenic bacteria.^[22] The antimicrobial activity of garlic is only due to thiosulfinates compound, so, garlic extract used in the treatment of asthma, arthritis, sciatica, lumbago, backache, bronchitis, chronic fever, tuberculosis, rhinitis, malaria, obstinate, skin disease including leprosy, leucoderma, discolouration, itches, indigestion, colic pain, enlargement of spleen, piles, fistula, fracture of bone, anemia, jaundice, epilepsy, contract and nightblindness, tumor ulcer, managing high cholesterol level, hysteria, dropsy, scurvy, vampires and act as powerful antioxidant. It detoxifies the body cleansing the kidney and increase the urine flow. During the past two decade more attention has been given to uncovering the benefits of garlic sulfur compound in relation to cancer. Some garlic constituents have been shown to alter activation of carcinogen and cause growth inhibition of tumor cells.

Allicin and wide range of other organosulfurate compounds which are known to be the constituents linked to the garlic properties. Allicin (2-propene-1-sulfinothionic acid 5-2-propenyl ester) is a thioester of sulfonic acid and exhibit antioxidant,^[23] antimicrobial,^[24-26] antiviral,^[27] antioxidant,^[23] anticancer,^[28] and has significant anticholesterol activity,^[29] so, used to prevent heart diseases including arteriosclerosis (hardening of arteries), high blood pressure, sugar, digestive disorder, reduce platelet aggregation, hyperlipidemia,^[30] reduce the

incidence of a multitude of chemically induced tumor and help for AIDS patient to treat cryptosporidium and toxoplasmosis, and used as antihypertensive.^[30] Allicin has a wide spectrum of antibacterial activity against numerous gram(+) and gram(-) bacteria such as *E. coli*, *salmonella enterica*, *Shigella*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Candida albicans* and *Streptoproteus*.^[31-37] Pharmacokinetic studies indicate that allicin will reach a maximum level in the blood after 30-60 minutes and may still be present 72 hours later with more than 85% clearance through urine and faecal path-way.^[38] Similarly allicin have significant enhancing effects on the immune system. It has been reported that the antibiotic activity of 1 mg of allicin is equated to that of 15 IU of penicillin.^[39]

In the present communication we deal to highlight the antimicrobial activity of the allicin at different concentration, temperature and pH against different bacteria and fungi by using standard method, along MIC, activity index.

EXPERIMENT

Chemical composition of *allium sativum*

The chemistry of garlic do not fully understand because each tiny clove contains the potential for almost 200 chemicals, that can be generated and interact with each other in a number of ways but the major components are as sulfur compound (alliin, allicin and ajoene), volatile oil, enzyme (allinase, peroxidase and miracynase, bioflavonoids (quercetin, cyaniding, allistain-I and II) carbohydrates (glucose and sucrose), protein, saponine, allinase enzyme, vitamin A, B, B₃, B₅, B₆, C, E, minerals such as Se, Ca, Fe, Mg, Mn, K, Na, Zn, amino acids (cystein, glutamine isoleucine and methionine), flavonoids, scardinine and antioxidant.

Isolation of Allicin

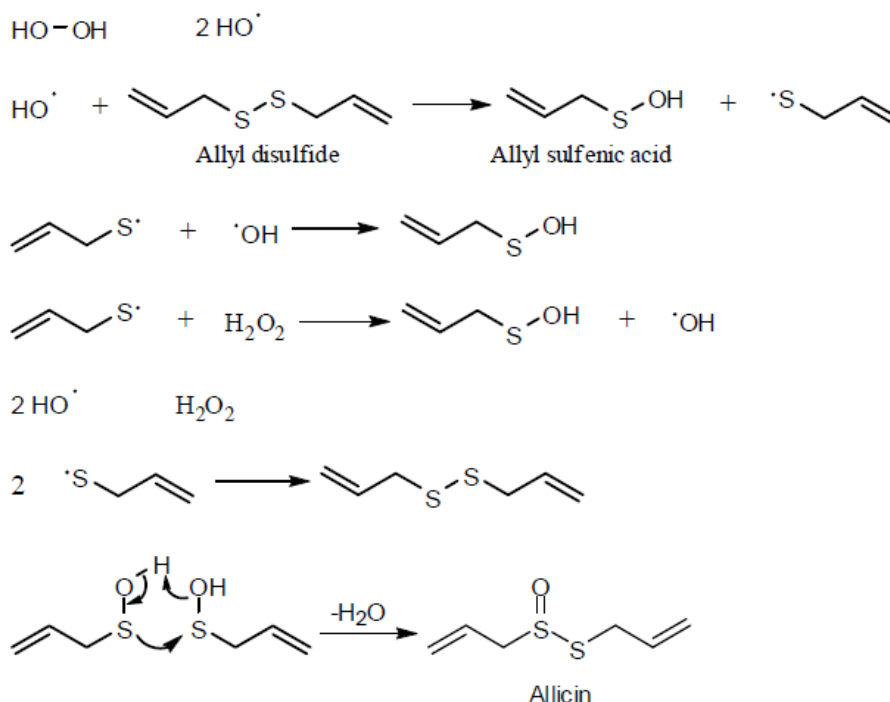
Allicin (2-propene-1-sulfinothinic acid S-2-propenyl ester or diallyl thiosulfinate) is a bright yellow oily liquid with a characteristic garlic odour.^[40] It is very unstable and can be stabilized with preserved pharmacological activity by forming inclusion complexes with β -cyclodextrins.^[41]

Naturally allicin (diallyl thiosulfinate) does not occur in *allium sativum*, instead it is found in the form of the amino acid- alliin(S-allyl cystein sulphoxide). When it is crushed, the alliin reacts with phosphopyridoxal enzyme allinase located in separate parts of garlic clove.^[42] In this transformation an unstable alliin- allinase complex is formed, which is further subjected to

dehydration by pyridoxal phosphate and transformed to allyl sulfenic acid, pyruvic acid and ammonia.^[43] Allyl sulfenic acid transformed to allicin by self condensation. Complete transformation occur in 10 -15 minutes at optimum temperature (33°C) and pH (6.5). Allinase enzyme become inactive in acidic medium, so this transformation can be inhibited upto 90% in the presence of retinol and hydroxyl amine sulfate solution by blocking the flavin components of this enzyme. Allinase is irreversibly deactivated below a pH of 3, so allicin is generally not produced in the body by the consumption of allium sativum.

Synthesis of allicin: Allicin was synthesized by standard protocols.^[44-47]

Mechanism: Due to the sufficient instability of allicin, transformation was proposed by free radical mechanism.^[48]



Formation of hydroxyl free radical was supported by bond dissociation energy of O-O bond (200.9 kJ/mol) in peroxide which is lower than S-S bond (301.39 kJ/mol), favors attacks allyl disulfide molecule to produce thiyl-radical and allyl sulfenic acid. The thiyl-radical can be further combined with hydroxyl-radical to give an unstable allyl sulfenic acid, or it can be reacts with the non-degraded hydrogen peroxide to produce a new hydroxyl-radical and allyl sulfenic acid. Also, allyl disulfide can formed by recombination of two thiyl-radicals, while two molecules of allyl sulfenic acid transformed to allicin with the elimination of water

molecule. This transformation is of zero order because the concentration of compound in slowest step is constant,^[48] and depend upon solvents and temperature. Maximum transformation take place in acetonitrile while minimum transformation take place in chloroform and most significant change of allicin concentration occurs in first five days in all solvent used and the rate of transformation of allicin significantly faster at higher temperature than at room temperature and most significant transformation of allicin occurs within two days.

ANTIMICROBIAL STUDIES

a. Antibacterial screening

The antibacterial activity of the test compound were measured by paper disc diffusion method, using agar nutrient medium and 5 mm diameter paper discs of whatman No.1 filter paper discs were soaked in a solution of known amount (0.4 to 0.9% w/v) of test compound dried and laid on the surface of petri-plates which were already seeded with the test organism. All the agar dishes were then incubated in an incubator at $27 \pm 1^\circ\text{C}$ for about 48 hours. After the incubation period, the growth of the microorganism was studied as inhibition zone (mm), around each disc in the form of turbid layer, except in the region where the concentration of antibacterial agent is above the MIC and zone of inhibition is seen. The size of the zone of inhibition depends upon sensitivity of the organism, nature of the culture medium, incubation condition, rate of diffusion of the agent and the concentration of the antibacterial agent on the filter paper.

b. Antifungal screening

The antifungal activity of different concentrations (0.40 to 0.90% w/v) of test compound was measured by determining the growth of test fungi by dry weight increase method and Richard liquid medium used as culture medium.^[49] The test compounds of varying concentration (0.40 to 0.90% w/v) were directly added in a Richard liquid medium having interested fungus in a sterilized chamber and was kept for seven days in an incubation chamber at $27 \pm 1^\circ\text{C}$. Media with test solution served as treated while without them as check. The resultant mycelial mats in each set were carefully removed, washed, dried and then weighed separately. The percentage of inhibition was calculated by the following formula

$$\text{Percentage inhibition of fungal growth} = \frac{(C_g - T_g) \times 100}{C_g}$$

where, C_g = Average growth in the check set

T_g = Average growth in the treated set

c. Effect of pH and temperature on antimicrobial activity of allicin

The effect of pH on the antimicrobial activity of allicin at room temperature was tested by adjusting pH with 1N NaOH and / or 1N HCl and allowed to stand for 4 hrs at room temperature. The activity of allicin was tested by paper disc agar diffusion method and by dry weight increase method. The thermal stability of allicin has also been determined at different temperatures.

RESULT AND DISCUSSION

Antimicrobial Activities

Allicin is considered to be the most potent antibacterial agent in crushed garlic extract and used to treat methicillin resistant staphylococcus aureus(MRSA) that have become resistant to all penicillin drugs, like methicillin, oxacillin and amoxicillin. Allicin manifests a wide spectrum of antibacterial activity against numerous gram(+)ve and gram(-)ve bacteria and fungus such as *Escherichia Coli*, *Salmonelenterica*, *Shigella*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Streptoproteus*, *Candida albicans*,^[50-57] *aspergillus flavus*, *aspergillus niger* and *cryptococcus neoformans*.^[26] Cyclodextrin complex of allicin exhibit more antimicrobial activity than allicin against *Staphylococcus aureus*, *Escherichia Coli*, *Pseudomonas aeruginosa* bacteria and *Candida albicans* and *aspergillus niger*. Allicin is recommended as co-therapy for the infections causes by *Mycobacterium tuberculosis*, an acid-resistant bacterium.^[58]

The fungicidal and bactericidal data of the graded concentrations of allicin against different bacteria and fungus at different temperature were recorded in the table [1-6] Fig. [1-9].

The observed results reveal that the antimicrobial activity of the compound is directly proportional to the concentration of the test compound and differ from fungus to fungus and bacteria to bacteria. The antibacterial outcome of allicin is of a broad spectrum. In most cases the 50% lethal dose concentration were same what higher than those vital for some of the newer antibiotics. It has been noted that various bacterial strain resistant to antibiotics such as methicillin resistant staphylococcus aureus [causes eczema and acne] as well as multi drug resistant enterotoxigenic strains of *Echerichia Coli*, *Enterococcus* etc have been found to be allicin sensitive.

Table 1: Antimicrobial activity data of different concentrations of allicin against different bacteria at 27^oC.

S.No.	Test of organism	0.40%	0.45%	0.50%	0.55%	0.65%	0.75%	0.85%	0.90 %
1.	<i>S.Typhimurium</i>	-	8	10	15	25	35	46	52
2.	<i>Klebsiella pneumonia</i>	7	9	12	18	27	37	48	54
3.	<i>S. Typhi</i>	7	8	10	19	29	38	50	57
4.	<i>A. Hydrophila</i>		8	09	14	24	29	32	37
5.	<i>Chromobacterium Violaceum</i>	7	9	10	18	31	36	44	50
6.	<i>Enterobacter Faecalis</i>	8	10	12	20	28	37	46	52
7.	<i>S.Senftenberg</i>	7	10	13	22	24	40	48	54
8.	<i>Bacillus subtilis</i>	9	12	18	27	31	46	52	58
9.	<i>Streptoproteus</i>	7	8	09	17	24	32	42	48

Table 2: Antimicrobial activity data of different concentrations of allicin against different bacteria at 32^oC.

S.No.	Test of organism	0.40%	0.45%	0.50%	0.55%	0.65%	0.75%	0.85%	0.90%
1.	<i>S.Typhimurium</i>	-	7	9	13	19	28	37	45
2.	<i>Klebsiella pneumonia</i>		7	11	16	24	31	39	48
3.	<i>S. Typhi</i>		7	8	14	22	32	42	50
4.	<i>A. Hydrophila</i>		8	9	12	19	28	30	38
5.	<i>Chromobacterium Violaceum</i>	7	8	9	13	21	30	36	42
6.	<i>Enterobacter Faecalis</i>	7	9	10	15	22	34	38	45
7.	<i>S.Senftenberg</i>	7	9	11	17	25	32	41	47
8.	<i>Bacillus subtilis</i>	8	11	14	21	28	36	46	52
9.	<i>Streptoproteus</i>		7	8	12	19	24	31	37

Table 3: Antimicrobial activity data of different concentrations of allicin against different bacteria at 37^oC.

S.No.	Test of organism	0.40%	0.45%	0.50%	0.55%	0.65%	0.75%	0.85%	0.90%
1.	<i>S.Typhimurium</i>	-	-	6	8	11	14	18	22
2.	<i>Klebsiella pneumonia</i>	-	6	7	10	14	16	21	32
3.	<i>S. Typhi</i>	-	6	7	9	12	15	19	22
4.	<i>A. Hydrophila</i>	-	7	8	9	13	17	23	26
5.	<i>Chromobacterium Violaceum</i>	-	7	8	10	16	21	29	33
6.	<i>Enterobacter Faecalis</i>	-	8	9	11	17	23	31	34
7.	<i>S.Senftenberg</i>	-	8	10	12	18	27	35	42
8.	<i>Bacillus subtilis</i>	-	7	9	11	17	26	33	41
9.	<i>Streptoproteus</i>	-	6	8	10	15	20	27	32

It has been noted that the affinity of allicin was effected by the pH of the medium and temperature. The maximum zone of inhibition has been noted at the original pH against all tested microbes, while moderate activity has been observed at the neutral pH, results reveals that antimicrobial activity of allicin decreases with the increase of the pH value, which are accordace with Tynecka et al.^[59]

Table 4: Antifungal Activity Data of Allicin against different fungus at 27°C.

Conc.	% of inhibition	Aspergillus flavus		Aspergillus niger		Cryptococcus neoformans		Alternaria alternate	
		Control	Allicin	Control	Allicin	Control	Allicin	Control	Allicin
0.40%	Wt	1.040	0.97	1.00	0.94	1.01	0.95	1.068	0.99
	%		5.92		6.04		6.28		6.96
0.50%	Wt	1.038	0.952	0.96	0.874	0.98	0.89	1.064	0.96
	%		8.24		8.96		9.12		9.84
0.60%	Wt	1.035	0.92	0.90	0.79	0.94	0.83	1.060	0.93
	%		10.96		11.74		12.00		12.56
0.70%	Wt	1.030	0.88	0.82	0.70	0.86	0.73	1.048	0.89
	%		13.54		14.32		14.96		15.28
0.80%	Wt	1.028	0.86	0.74	0.615	0.78	0.643	1.032	0.846
	%		16.18		16.96		17.56		18.00
0.90%	Wt	1.020	0.826	0.68	0.55	0.66	0.52	1.020	0.80
	%		19.00		19.30		20.46		21.12

Table 5: Antifungal Activity Data of Allicin against different fungus at 32°C.

Conc.	% of inhibition	Aspergillus flavus		Aspergillus niger		Cryptococcus neoformans		Alternaria alternate	
		Control	Allicin	Control	Allicin	Control	Allicin	Control	Allicin
0.40%	Wt	1.038	0.978	1.002	0.943	1.016	0.953	1.064	0.99
	%		5.78		5.96		6.14		6.86
0.50%	Wt	1.034	0.95	0.95	0.87	1.014	0.92	1.062	0.96
	%		8.12		8.68		9.12		9.52
0.60%	Wt	1.030	0.92	0.92	0.815	0.91	0.89	1.052	0.92
	%		10.76		11.38		11.96		12.32
0.70%	Wt	1.024	0.89	0.82	0.705	0.96	0.82	1.040	0.88
	%		13.28		13.92		14.52		14.96
0.80%	Wt	1.018	0.86	0.76	0.635	0.89	0.737	1.038	0.853
	%		15.76		16.52		17.16		17.76
0.90%	Wt	1.012	0.824	0.64	0.522	0.82	0.656	1.024	0.22
	%		18.58		18.38		19.96		20.62

Table 6: Antifungal Activity Data of Allicin against different fungus at 37°C.

		Aspergillus flavus		Aspergillus niger		Cryptococcus neoformans		Alternaria alternata	
Conc.	% of inhibition	Control	Allicin	Control	Allicin	Control	Allicin	Control	Allicin
0.40%	Wt	1.034	0.98	0.97	0.913	1.046	0.983	1.058	0.786
	%		5.54		5.88		6.05		6.84
0.50%	Wt	1.028	0.946	0.93	0.85	1.042	0.95	1.052	0.952
	%		7.96		8.58		9.08		9.46
0.60%	Wt	1.020	0.912	0.86	0.764	1.036	0.915	1.046	0.918
	%		10.56		11.24		11.68		12.16
0.70%	Wt	1.00	0.87	0.80	0.69	1.028	0.878	1.038	0.885
	%		13.06		13.78		14.38		14.78
0.80%	Wt	0.98	0.828	0.72	0.602	1.04	0.86	1.026	0.845
	%		15.58		16.36		16.94		17.57
0.90%	Wt	0.96	0.785	0.62	0.506	0.96	0.77	1.00	0.80
	%		18.26		18.32		19.68		20.28

Table 7: Effect of pH on the antimicrobial activity of allicin.

S.No.	Test organism	Diameter of inhibition zone(mm) at different pH					
		6.0	6.5	7.0	7.5	8.0	8.5
1.	<i>S.Typhimurium</i>	24	21	17	13	10	09
2.	<i>Klebsiella pneumonia</i>	32	25	21	18	15	12
3.	<i>S. Typhi</i>	40	32	29	26	22	17
4.	<i>A. Hydrophila</i>	16	13	11	10	08	07
5.	<i>Chromobacterium Violaceum</i>	22	19	16	11	09	08
6.	<i>Enterobacter Faecalis</i>	29	24	19	15	12	10
7.	<i>S.Senflenberg</i>	36	28	25	21	19	15
8.	<i>Bacillus subtilis</i>	42	37	35	31	26	20
9.	<i>Streptoproteus</i>	18	15	13	11	09	08

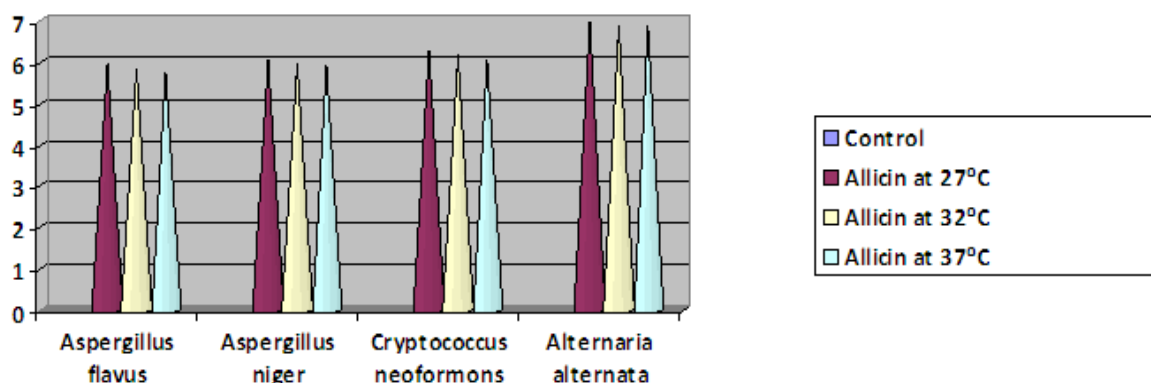


Fig.1: Antifungal Activity of Allicin against different fungus at 0.40 %.

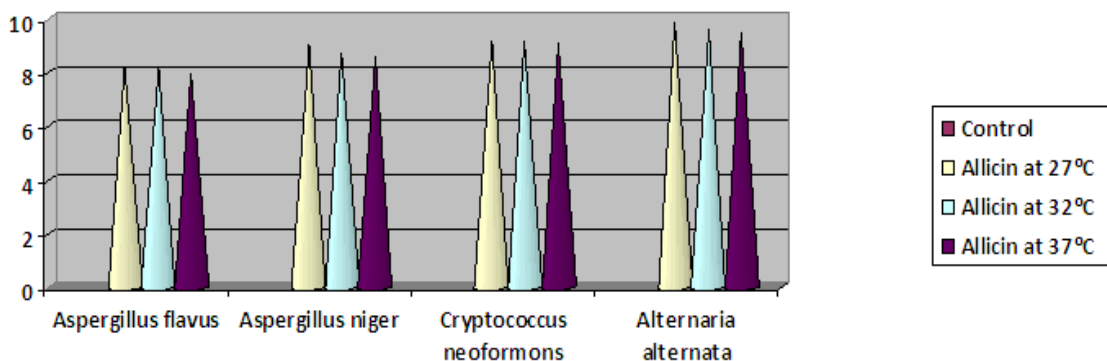


Fig. 2: Antifungal Activity of Allicin against different fungus at 0.50%.

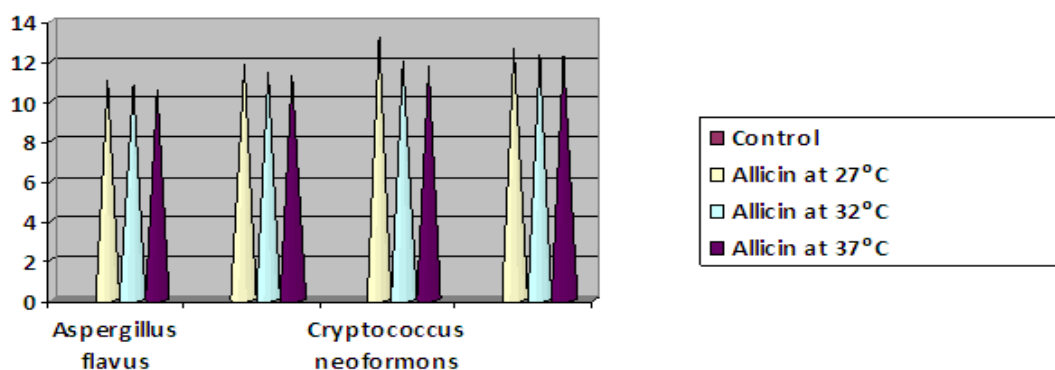


Fig. 3: Antifungal Activity of Allicin against different fungus at 0.60 %.

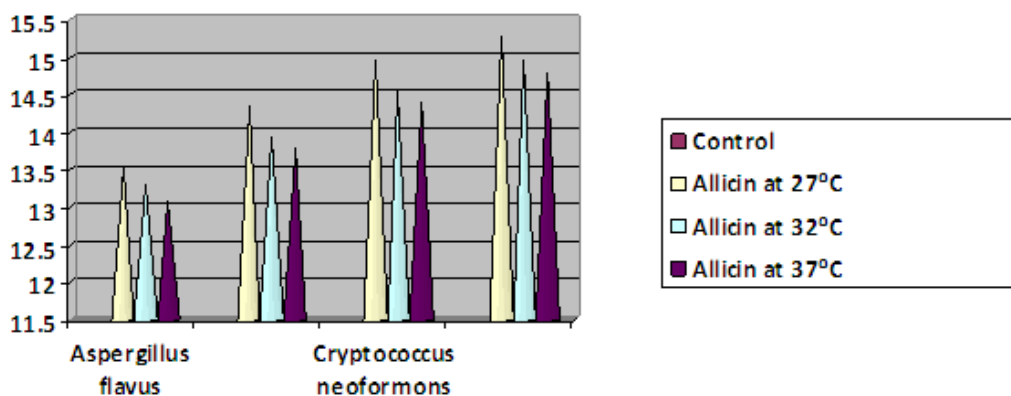


Fig. 4: Antifungal Activity of Allicin against different fungus at 0.70 %.

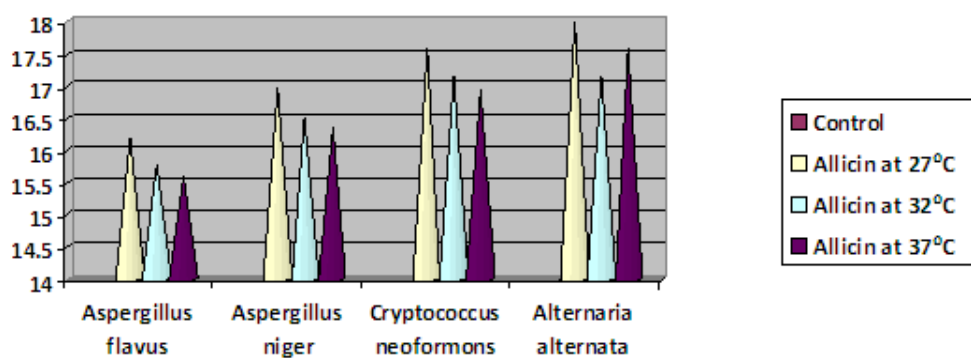


Fig. 5: Antifungal Activity of Allicin against different fungus at 0.80 %.

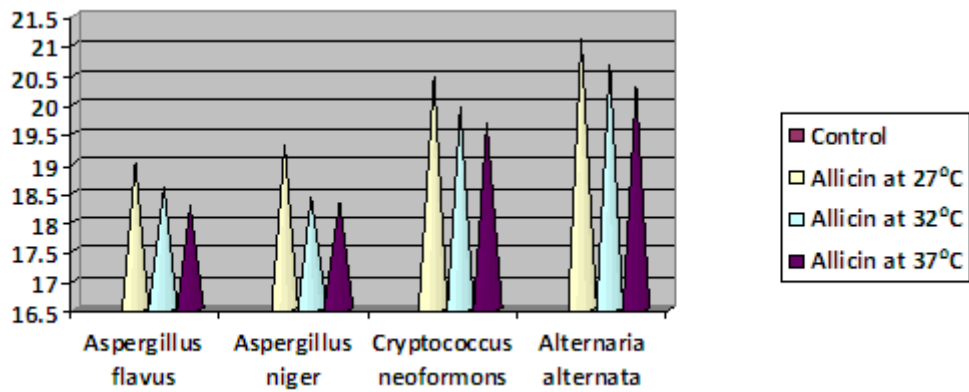


Fig. 6: Antifungal Activity of Allicin against different fungus at 0.90 %.

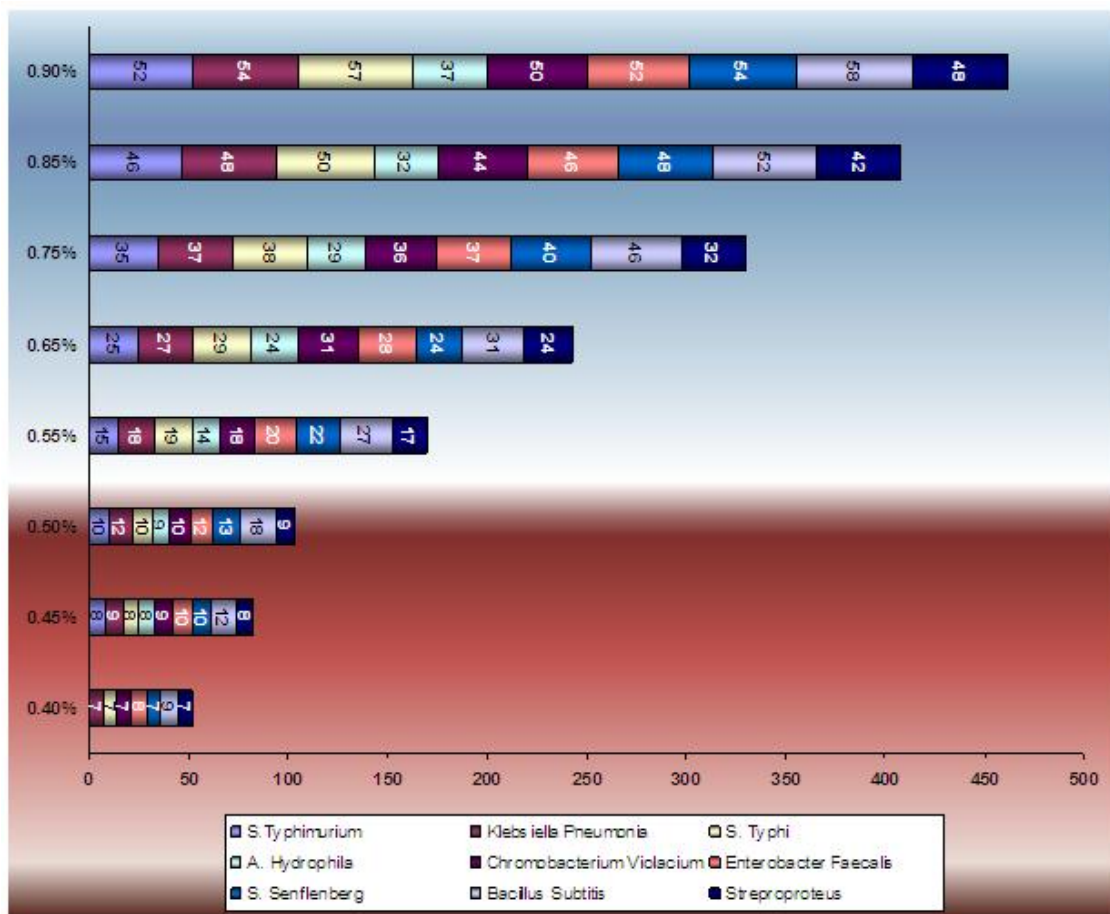


Fig 7: Antibacterial Activity of Allicin against different bacteria at different conc. At 27°C.

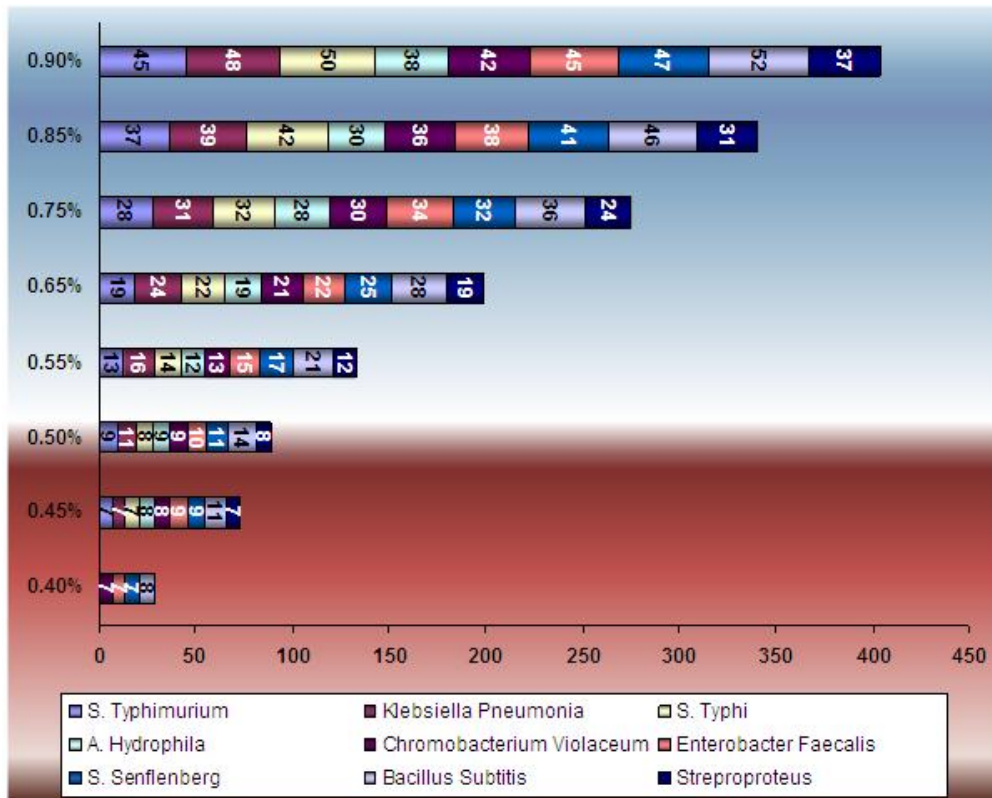


Fig. 8: Antibacterial Activity of Allicin against different bacteria at different conc. At 32°C.

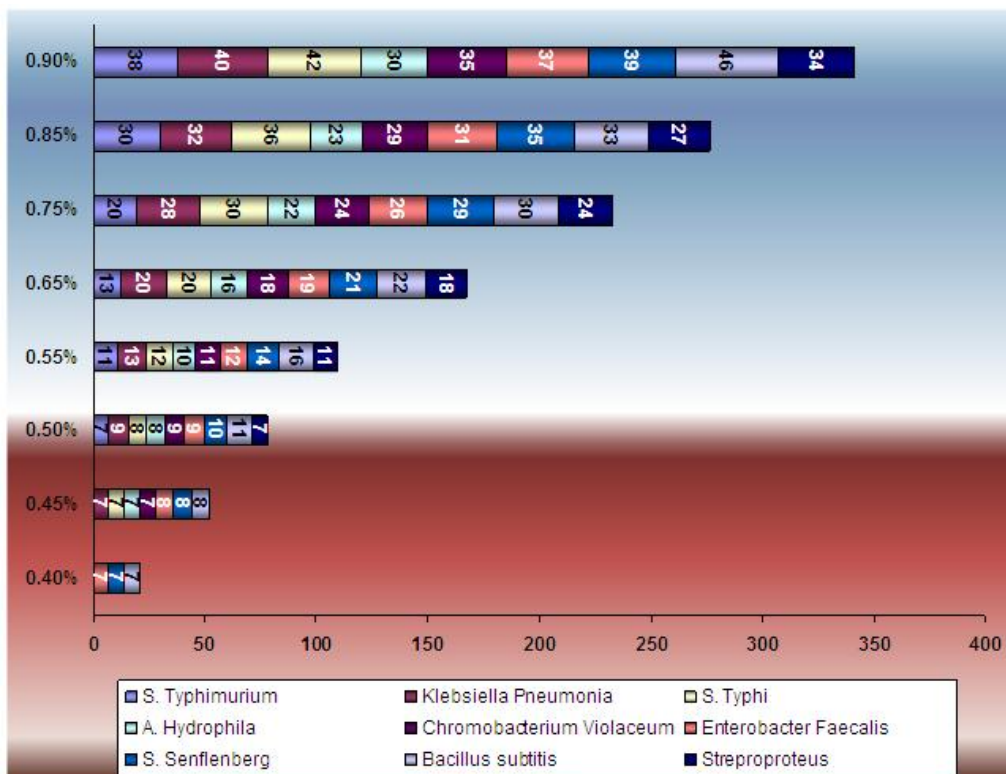


Fig. 9: Antibacterial Activity of Allicin against different bacteria at different conc at 37°C.

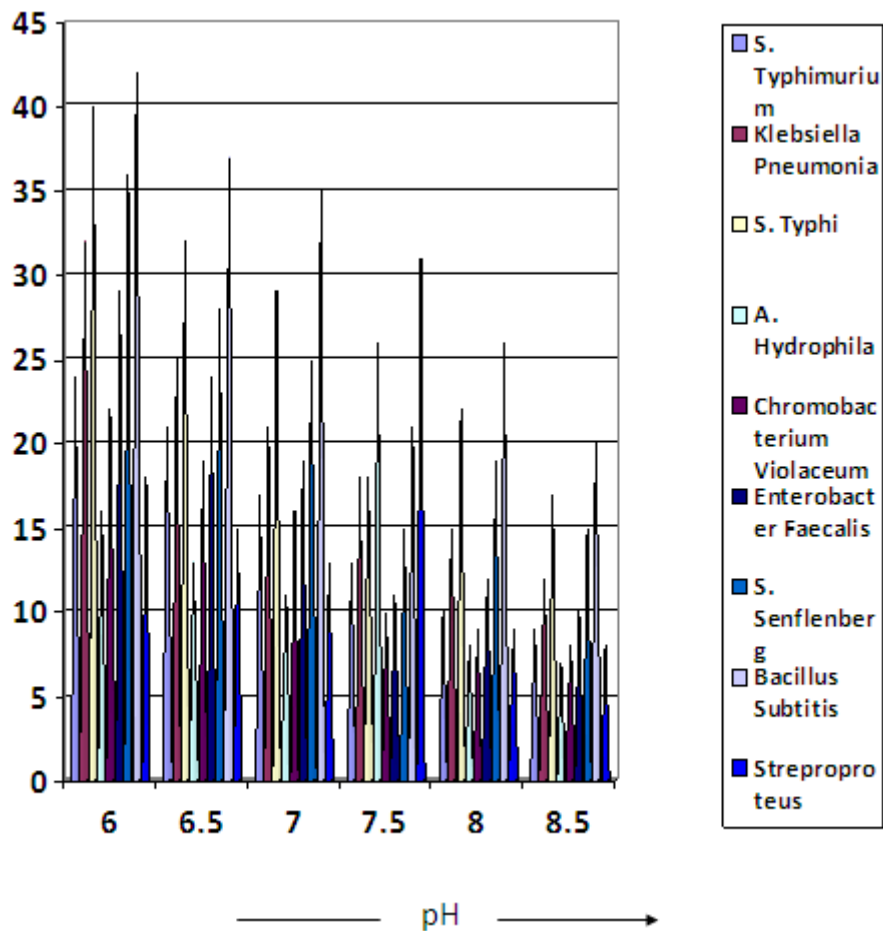


Fig. 10: Effect of pH on the antimicrobial activity of allicin.

Antimicrobial activity of allicin also effected by temperature, its values decreases with the increase of temperature, and completely destroyed at 80⁰C to 90⁰C, which was supported by several researcher.^[60-61]

Mechanism—Antimicrobial agents interfere chemically with the synthesis of function of vital components of microorganism in the different ways. Inhibitors of cell wall synthesis, inhibitors of cell membrane, inhibitors of biosynthesis (i.e. production of purines, pyrimidine, A.A., Vitamins, protein, DNA, RNA), inhibitors of energy production (inhibit the respiration or by uncoupling of oxidative phosphorylation).

The biological activity of allicin is to be related to a combination of following factors.

1. Its activity as an antioxidant.
2. Its ability to attack the sulphur [SH] group in enzymes and proteins and modify their activities.
3. Its ability to rapidly penetrate into cells through the cell membrane.

Antimicrobial activity of allicin is mainly due to S-S and S-O bond which has the ability to react with thiol containing enzyme[L-cysteine] to form the S-thiolation product S-allyl mercaptocysteine which is characterized by NMR and Mass spectroscopy. It has been noted that in amoeba parasite, allicin was found to strongly inhibit the cysteine proteinases, alcohol hydrogenases.^[53]

Inhibition of these enzymes were noted at rather low concentrations [$< 10 \mu\text{g/ml}$]. Allicin also irreversibly subdued the well known thio-protease papain.

Allicin also inhibits other bacterial enzymes such as the acetyl-co-A forming system consisting of acetate kinase and phosphotransacetyl-co-A synthetase.^[54] Allicin was found to partially inhibit the DNA and protein synthesis but the effect on RNA was immediate, suggesting that this could be a primary target of allicin action.^[55] The mechanism of antifungal effect in lower eukaryotes is considered to be inhibition of phosphotidyl chloride biosynthesis.^[56]

It conclude that the broad spectrum antimicrobial effects of allicin is due to the multiple inhibitory effects on various thiol dependent enzymatic systems. It could be noted that allicin effect is not same for all target. Thiol protease could be inhibited at the lowest concentrations.

CONCLUSION

A lot of antimicrobial agents have been discovered so far but the multi- drug resistance phenomenon is a major obstacle to the use of antimicrobial agents. This promoted research to discover some novel antimicrobial agents from medicinal plants so as to eliminate toxicity factor of single component antimicrobial drugs.

It has been concluded from this study that allicin appear to satisfy all the criteria as antimicrobial agents. With a large number of fungi and bacteria becoming resistant to antibiotic drugs, search for new antifungal and antibacterial agent is very essential in recent time. In view of the strong antifungal and antibacterial activity of allicin, the research should continue to modify the isolated allicin by chemical process to form more potent antifungal and antibacterial agents. Also, more dose-response preclinical studies as well as clinical studies need be carried to develop some noble drugs or their precursors.

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