SYNTHESIS, CHARACTERIZATION AND EVALUATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF TRIAZOLE DERIVATIVES OF GALLIC ACID

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ABSTRACT: Heterocycles bearing a symmetrical triazole represent an interesting class of compounds possessing a wide spectrum of biological activities such as anti-inflammatory, anticancer, antitubercular, antiviral and antimicrobial properties. In present study an attempt was made to synthesize compounds using propyl gallate and hydrazine hydrate as starting material. And following the scheme five different compounds using different aromatic substituents. Synthesized compounds were subjected to physical characterization and spectral analysis for structural confirmation. The compounds were than subjected to evaluation of antibacterial and antifungal activity. The bacterial screening indicated that among the test compounds number S_2, S_3, moderately activity against all the tested bacterial strains *Bacillus subtilus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*. The remaining compounds were found to be less active. Minimum inhibitory concentration value of the compounds against the bacteria stains reveals that compound S_3, S_4 have better onset of action against all bacteria strains as compared to that of standard.

Antifungal screening revealed that the test compounds showed moderate activity against *Aspergillus niger.*

Keywords: antibacterial activity, antifungal activity, triazole, gallic acid, propyl gallate.

INTRODUCTION:

Triazole is one of a class of organic heterocyclic compounds containing a five-membered diunsaturated ring structure composed of three nitrogen atoms and two carbon atoms at nonadjacent positions. The simplest member of the Triazole family is triazole itself, white to pale yellow crystalline solids with a weak characteristic odor; soluble in water and alcohol, melts at 120^0C, boils at 260^0C[1-4].

Heterocycles bearing a symmetrical triazole represent an interesting class of compounds possessing a wide spectrum of biological activities such as anti-inflammatory, anticancer, antitubercular, antiviral and antimicrobial properties. It has also been reported that derivatives of 1, 2, 4-triazole nucleus system found to have diverse pharmacological activities such as fungicidal, insecticidal, bactericidal, herbicidal, antitumor, anti-inflammatory, CNS stimulant properties. They also find applications as dyes, lubricants and analytical reagents, antiviral agents[6].

- Someone in the world is newly infected with TB bacilli every second.
- Overall, one-third of the world's population is currently infected with the TB bacillus.

- 5-10% of people who are infected with TB bacilli (but who are not infected with HIV) become sick or infectious at some time during their life. People with HIV and TB infection are much more likely to develop TB.

The triazole antifungal drugs include fluconazole, isavuconazole, itraconazole, voriconazole, pramiconazole, and posaconazole
The triazole plant protection fungicides include epoxiconazole, triadimenol, propiconazole, cyproconazole, tebuconazole, flusilazole and paclobutrazol, as well as the potent antiviral N-nucleoside ribavirin

MATERIAL AND METHOD:

All the chemicals were purchased from local market and purified according to established method. Melting points were recording using VEEGO digital melting point apparatus. The homogeneity and purity of synthesized compound was ascertained by using TLC, performed on silica gel G coated plates using ethyl acetate and pet. Ether (1:1) an eluent, the developed plates were observed under U.V light.
Perkin Elmer FT-IR spectrometer, Bruker advance II 400 MHz NMR spectrometer, LC-MSD-Tranp SL 2010 A SHIMADZU were used for structural elucidation of compounds.
The compounds were prepared according to established method shown in schematic diagram.

Step-I: Synthesis of 3, 4, 5-trihydroxybenzohydrazide(Galloyl hydrazide)

Propyl Gallate (0.01 moles) and hydrazine hydrate (0.01 moles) are mixed gently and refluxed for 6 hrs. and the mixture is then cooled and pour into ice cold water. Filtered off the crystals and recrystallized from ethanol. Completion of reaction was monitored on TLC using silica gel-G coated plates by using ethyl acetate and petroleum ether as the eluent and observed in U.V. light.

1 (a) Yield 74%, Melting point 134^0C

Step II: Synthesis of 2-[(3,4,5-trihydoxyphenyl)carbonyl]-*N*-hydrazine phenyl carbothioamide.

A mixture of 3, 4, 5-trihydroxybenzohydrazide (0.01 mol) and phenyl isothiocyanate (0.001 mol) in ethanol (25.0 ml) was refluxed on a water bath for 2 hrs. The solvent was concentrated and the precipitated product was filtered, dried and recrystallized from methanol. Completion of reaction was monitored on TLC using silica gel-G coated plates by using ethyl acetate and petroleum ether(1:1) as the eluent and observed in U.V. light.

2 (a) Yield 69%, Melting point 154^0C

Step III: Synthesis of 5-(4-amino-5-(phenylamino)-4*H*-1,2,4-triazol-3-yl)benzene-1,2,3-triol

Compound 2-[(3,4,5-trihydoxyphenyl) carbonyl–N-hydrazine phenyl carbothioamide (0.002 mol) and hydrazine hydrate (0.025 mol) was refluxed in methanol for 2 hrs. at a temperature between 50-60 ^{0}C, reaction mixture was cooled and poured over crushed ice. Solid was filtered and recyrastallized from methanol. The completion of reaction was monitored on TLC using silica gel-G coated plates by using ethyl acetate and petroleum ether(1:1) as the eluent and observed in U.V. light. Yield 71%, Melting point 167^0C

SCHEME[1-5]

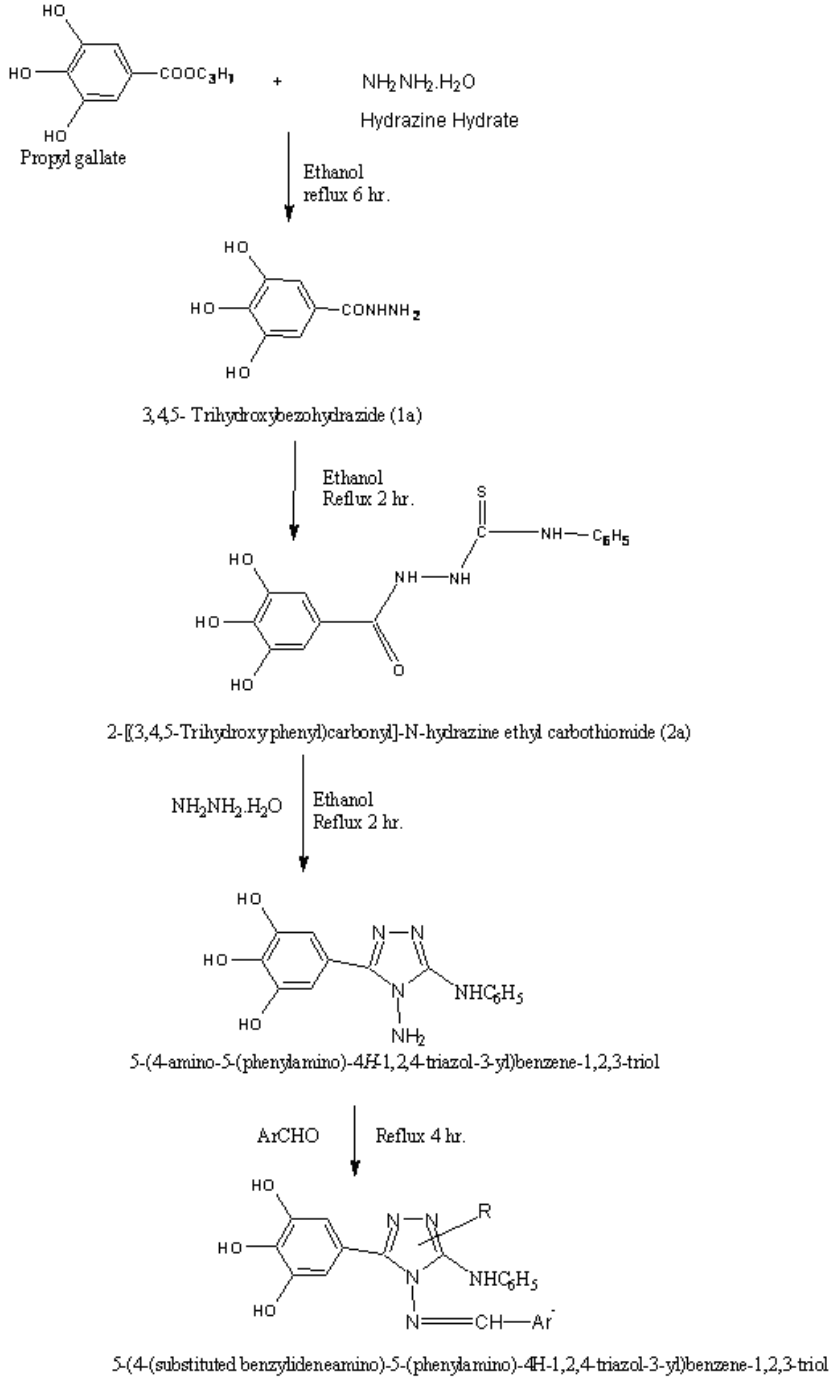
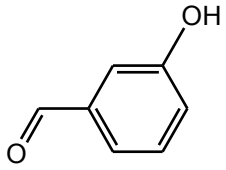
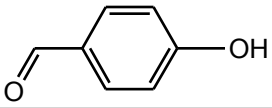
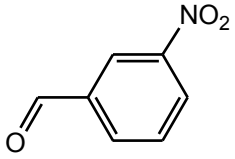
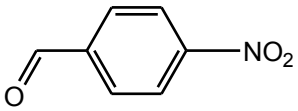
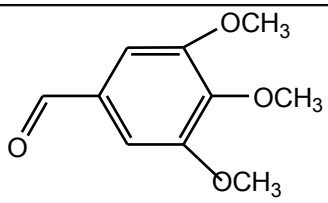

HO
HO
HO
COOC3H7
Propyl gallate
+
NH2NH2.H2O
Hydrazine Hydrate
Ethanol
reflux 6 hr.
CONHNH2
3,4,5- Trihydroxybezohydrazide (1a)
Ethanol
Reflux 2 hr.
S
C
NH—C6H5
NH—NH
O
2-[(3,4,5-Trihydroxyphenyl)carbonyl]-N-hydrazine ethyl carbothiomide (2a)
NH2NH2.H2O
Ethanol
Reflux 2 hr.
N—N
NHC6H5
NH2
5-(4-amino-5-(phenylamino)-4H-1,2,4-triazol-3-yl)benzene-1,2,3-triol
ArCHO
Reflux 4 hr.
R
N=CH—Ar
5-(4-(substituted benzylideneamino)-5-(phenylamino)-4H-1,2,4-triazol-3-yl)benzene-1,2,3-triol

Table 1: List of aromatic aldehyde substituents

S. NO.	COMPOUNDS	STRUCTURE
1.	m- hydroxy benzaldehyde	
2.	p- hydroxy benzaldehyde	
3.	m- nitro benzaldehyde	
4.	p-nitro benzaldehyde	
5.	3,4,5- trimethoxy benzaldehyde	

Step IV: Synthesis of 5-(4-(substituted benzylideneamino)-5-(phenylamino)-4H-1,2,4-triazol-3-yl)benzene-1,2,3-triol

To a solution of 5-(4-amino-5-(phenyl amino)-4H-1,2,4-triazol-3-yl)benzene-1,2,3-triol (0.01 mol) in absolute ethanol (30 ml), the appropriate aromatic aldehydes ().012 mol) was added. The reaction mixture was refluxed for 4 hrs. The formed solid after cooling was filtered off and recrystallized to give the title compounds respectively.

Physicochemical Parameters

Gallic acid derivatives containing triazole nucleus were synthesized and characterized by the following experimental methods.

- **Melting point** of the synthesized compounds was taken in open capillary tubes and was uncorrected and tabulated in Table 2.
- **Thin layer chromatography** was preformed using plates coated silica gel of 0.25 mm thickness. Eluents used ethyl acetate:petroleum ether. Spots were visualized through the iodine chamber. R_f values of the newly synthesized compounds were calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

- **Solubility:** At room temperature solubility of a newly synthesized compound were determined by various organic solvents audit was found that all the compounds are freely soluble in DMSO, ethanol, methanol and water.

Spectral Analysis

To confirm the chemical structures of the synthesized compounds, and the various functional groups in the final compounds spectral analysis was performed. (Table no.3)

- The Infra Red spectroscopy was performed with KBr on Perkin FT-IR instrument.
- ^{1}HNucler Magnetic Resonance spectroscopy was recorded on Bruker Advance II 400 MHz NMR Spectrometer. The chemical shifts were reported as parts per million downfield from tetramethylsilane, solvent used as Dimethyl sulfoxide.
- Mass spectroscopy was performed on LCMS 2010A using Dimethyl sulfoxide as solvent.

Antimicrobial and anti fungal Evaluation

The Various methods have been used to evaluate the antimicrobial activity of the drugs with their own advantage and limitations. The techniques involved in evaluation are,

- Agar streak dilution method
- Serial dilution method
- Agar diffusion method
  - Cup plate method
  - Paper cylinder method
  - Disc diffusion method
- Turbid metric method

The antibacterial activities of the compounds will study by using cup Plate method.

Media Used

Nutrient agar was used as the media for the study.

Nutrient agar composition

Ingredients	Gram/Liter
Peptic Digest of Animal Tissue	5.00
Beef Extract	3.00
Sodium Chloride	5.00
Agar	15.00

Antibacterial activity

In present study the following bacteria were used.

A. *Escherichia coli (Gram -ve)*
B. *Pseudomonas aeruginosa (Gram -ve)*
C. *Klebsiella pneumoniae (Gram +ve)*
D. *Staphylococcus aureus (Gram +ve)*

In present study the cup-plate method was used to evaluate the antimicrobial activity in vitro of the synthesized compounds. This method was used for determining the selective effectives of the anti bacterial activity. The standard antibiotic selected for study of the antibacterial activity was ciprofloxacin.

CUP-PLATE METHOD USING NUTRIENT AGAR[4, 8]:

Materials used[9,10]

- Nutrient agar, growth culture in 18-24hrs.
- Sterile Petri dishes
- Sterile pipettes
- Sterile cotton swabs
- Sterile cork borer
- Sterile test tubes containing the solution of the test compounds in desired concentrations.

Preparation of nutrient agar:

The definite volumes off peptone (0.6%), yeast extract (0.15%), dipotassium dihydrogen phosphate (0.36%) and potassium dihydrogen phosphate (0.13%) were dissolved in distilled water and pH was adjusted to 7.2. this solution was sterilized by autoclaving at 15 psi for 20 minutes.

Preparation of sub-culture

One day prior to this testing, inoculation of the above bacterial cultures were made in the nutrient agar and incubated at 37°C for 18-24 hrs.

Preparation of test solutions

Each test compound (5mg/ml) was dissolved in dimethyl sulfoxide (5ml) to give a 1,000 µg/ml.

Method of testing

Base layer was obtained by pouring about 10-15ml of the base layer medium into each sterilized Petri dishes and allowed to attain room temperature. The over night grown sub-culture was taken in to definite volume of inoculated, then with the help of cotton swab the organisms were streaked the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface. Allow all culture plates to dry for about 5 minutes.

Scooping out nutrient agar with sterilized cork borer made the cups. The solution of the test compounds (0.1ml) were added into the cups by using micropipettes and these plates were subsequently incubated all the plate cultures in an inverted position for 24 hours at 37 ^{0}C and observed for antimicrobial activity. Ciprofloxacin (10µg/ml) was used as standard drug and the solvent control (DMSO) was kept separately.

After 24 hrs, the diameters of zone of inhibition were measured for the plates in which the zones of inhibition and minimum inhibitory concentration (MICs) were measured in mm for each organism.

Zone of inhibition were determined for all the ten compounds the results in the form of per cent inhibition were summarized in below the tables.(table no. 5)

The minimum inhibitory concentrations (MIC) of the synthesized compounds are tabulated in table no. 6.

Antifungal activity[4, 8]

The synthesized compounds were screened for their antifungal activity against *Aspergillus niger*. By cup-plate method, Nutrient agar media was prepared by dissolving in distilled water. Twenty milliliters of agar media was poured into each Petri dish and plates ensure that the layer of the medium are uniform in thickness, by placing the plates on a level surface and plates were dried, *a* loopful of *Aspergillus niger* strain was inoculated into nutrient agar media of each plate, and incubated at 25±2ºc for 72 hours. Ketoconazole was used as standard drug.

Using a punch, wells were made on these seeded agar plates minimum inhibitory concentrations of the test compounds dissolved in 100% dimethyl sulfoxide (DMSO), at three different concentration 100µg/ml, 200µg/ml and 300µg/ml for all the test compounds, were added into each labeled well. A control was also prepared for the plates in the same way using solvent DMSO.

The zone of inhibition and minimum inhibitory concentrations (MICs) were determined in comparison with the standard drug Ketoconazole. Ketoconazole has an inhibition length of 24 mm at 10 µg/ml concentration. Zone of inhibition were determined for all the ten compounds the results in the form of per cent inhibition were summarized in below table along with the standard and the MIC values. (table no.7 & 8)

RESULTS AND DISCUSSION:

Conclusion:

The compounds (S_1-S_5) were synthesized and structure was determined by different spectral analysis. The compounds were than subjected to evaluation of its antibacterial and antifungal activity. The antimicrobial activity revealed that some of the test compounds showed moderate inhibition at 100 µg/ml, 200 µg/ml and 300 µg/ml concentration. The bacterial screening indicated that among the test compounds number S_2, S_3, moderately activity against all the tested bacterial strains *Bacillus subtilus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*. The remaining compounds were found to less active. Minimum inhibitory concentration value of the compounds against the bacteria stains reveals that compound S_3, S_4 have better onset of action against all bacteria stains as compared to that of standard.

Antifungal screening revealed that the test compounds showed moderate activity against *Aspergillus niger.*

Physical characterization

Table2 :Physical parameters of synthesized compounds

S. No.	Code of compound	R_f value	Melting point (^{0}C)	Appearance	% of yield	Molecular formula	Molecular weight (gm)
1.	S_1	0.64	159^{0}C	Brown	80	$C_{24}H_{23}N_5O_6$	477.47
2.	S_2	0.68	169^{0}C	Yellow	69	$C_{21}H_{16}N_6O_5$	432.39
3.	S_3	0.83	179^{0}C	Orange	63	$C_{21}H_{16}N_6O_5$	432.39
4.	S_4	0.69	187^{0}C	Brown	77	$C_{21}H_{17}N_5O_4$	403.39
5.	S_5	0.75	197^{0}C	Yellow	79	$C_{21}H_{17}N_5O_4$	403.39

Spectral analysis

Table3:Spectral data of synthesized compounds

S. No.	Compounds Code	NMR Data	IR Data
1.	S_1	3.65 (s, 9H, CH_3),4.20(s, 1H, NH),5.10 (s, 3H, OH), 6.2-6.75 (m, 9H, Ar-H),8.2(s, 1H, CH)	O-H stretching 3415,=C-H stretching 3158, -C-H stretching 2981,C-O stretching1036, C=C stretching 1658,C=N stretching 2353 C-N stretching 1093
2.	S_2	4.15 (s, 1H, NH) 5.2 (s, 3H, OH) 6.2-6.8 (m, 11H, Ar-H) 8.2 (s, 1H, CH)	O-H stretching 3458, =C-H stretching 3192, C=N stretching 2333, C-O stretching 1104 , =C stretching 1660, C-N stretching1153, C-NO_2 stretching 1383
3.	S_3	4.15 (s, 1H, NH) 5.20 (s, 3H, OH) 6.1-6.9 (m, 11H, Ar-H), 8.1 (s, 1H, CH)	O-H stretching 3295,=C-H stretching 3142, C=N stretching 2347,C-O , stretching 1016, C=C stretching 1613, C-N stretching 1044,C-NO_2 stretching 1323
4.	S_4	4.1 (s, 1H, NH), 5.2 (s, 4H, OH) 6.2-6.85 (m, 11H, Ar-H), 8.10 (s, 1H, CH)	O-H stretching 3458, =C-H stretching 3153, C=N stretching 2342, C-O stretching 1104, C=C stretching 1660, C-N stretching 1153
5.	S_5	4.20 (s, 1H, NH), 5.10 (s, 4H, OH) 6.2-6.75 (m, 11H, Ar-H) 8.2 (s, 1H, CH)	O-H stretching 3365, =C-H stretching 3116, C=N stretching 2362, C-O stretching 1016, C=C stretching 1613, C-N stretching 1044

Table 5: Antibacterial activity of synthesized compounds

Compound No.	Diameter of zone of inhibition in mm											
	E.coli			*S.aureus*			*B.subtilus*			*K.pneumonea*		
	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml
S_6	12	13	15	13	16	16	12	13	14	12	14	15
S_7	11	12	13	12	14	15	15	16	16	11	13	14
S_8	13	13	15	12	13	14	12	14	14	13	14	15
S_9	13	14	15	12	14	15	14	15	16	12	13	14
S_{10}	11	12	13	12	13	14	13	14	15	12	13	14
DMSO	—	—	—	—	—	—	—	—	—	—	—	—
Standard	22	22	22	20	20	20	21	21	21	22	22	22

Table 4: Structure of synthesized compounds

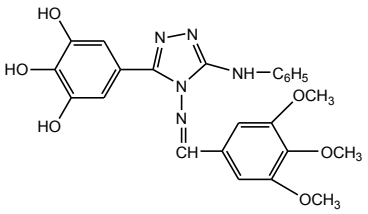
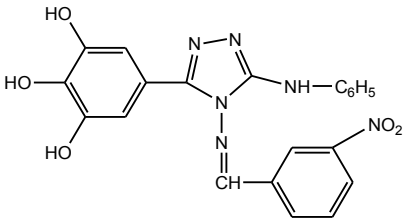
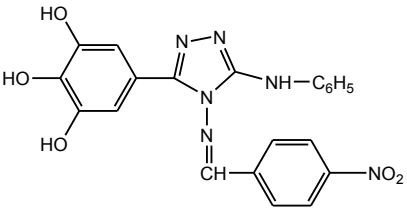
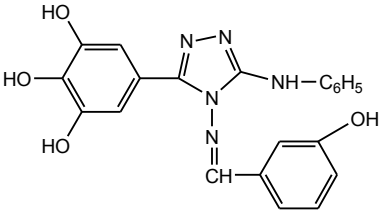
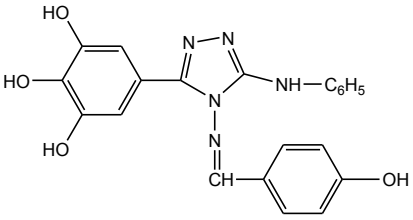
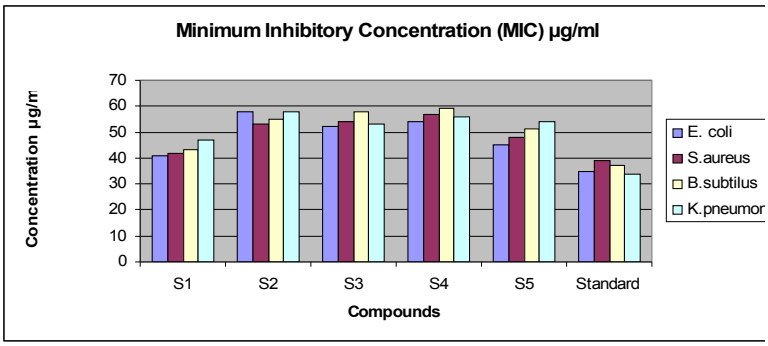
S.no.	Compounds	Structure of the Compounds
6	S_1	
7	S_2	
8	S_3	
9	S_4	
10	S_5	

Table 6: Antibacterial activity data for MIC

compounds	Minimum Inhibitory Concentration(MIC) in µg/ml			
	E.coli	*S.aureus*	*B.subtilus*	*K.pneumonea*
S_1	41	42	43	47
S_2	58	53	55	58
S_3	52	54	58	53
S_4	54	57	59	56
S_5	45	48	51	54
Standard	35	39	37	34

Ciprofloxacin 10µg/mL used as standard against *E.coli, Kpneumonae, S.aureus, P. Aeruginosa*



Anti bacterial activity

The results of antibacterial activity was tabulated at three different dose level i.e 100µg/ml - 300 µg/ml

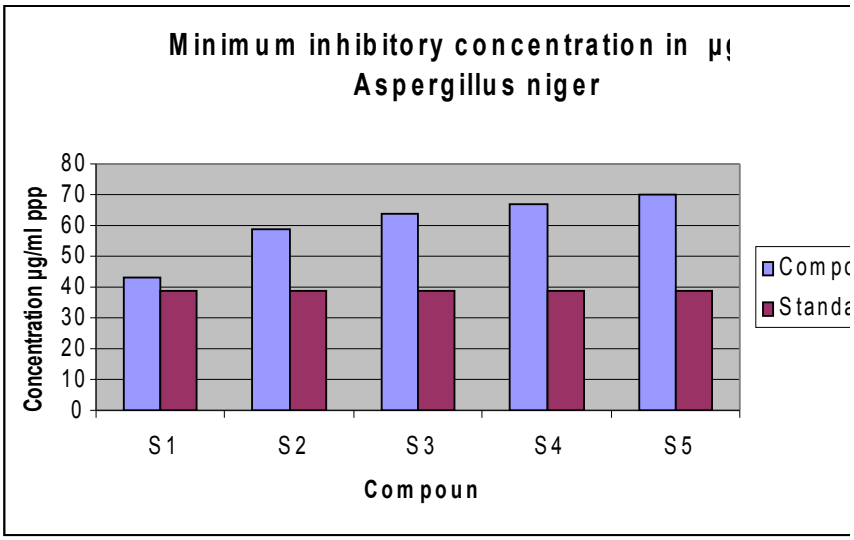
Table 7:–Antifungal activity against *Aspergillus niger*

Concentration in µg/ml	Diameter Of the Inhibition Zone (mm)					
	S_1	S_2	S_3	S_4	S_5	Std
100	12	11	10	11	13	24
200	14	13	13	13	14	24
300	15	15	15	14	16	24

Ketoconazole 10µg/mL used as standard drug against *A.niger.*

Table 8: Minimal inhibitory concentration (MIC) of compounds S_1-S_{10} in µg/ml

Compound No.	Minimal inhibitory concentration in µg/ml *(Aspergillus niger)*
S_1	43
S_2	59
S_3	64
S_4	67
S_5	70
DMSO	-
Standard	39



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