"Molecular sieves and ultrasound assisted synthesis of Novel1,3,4-oxadiazole-2-thione derivatives as potential antifungal Agents."

Presented By

Dr. Anna Pratima G. Nikalje [1] * ,
Ms Urja D. Nimbalkar [2],
Dr. Santosh Tupe[3] ,
Dr. Julio A. Seijas Vazquez [4], Dr. Maria Pilar Vazquez Tato[4]

1. Department of Pharmaceutical Chemistry, Dr. Rafiq Zakaria Campus, Y. B. Chavan College of Pharmacy, Aurangabad. MS. India .

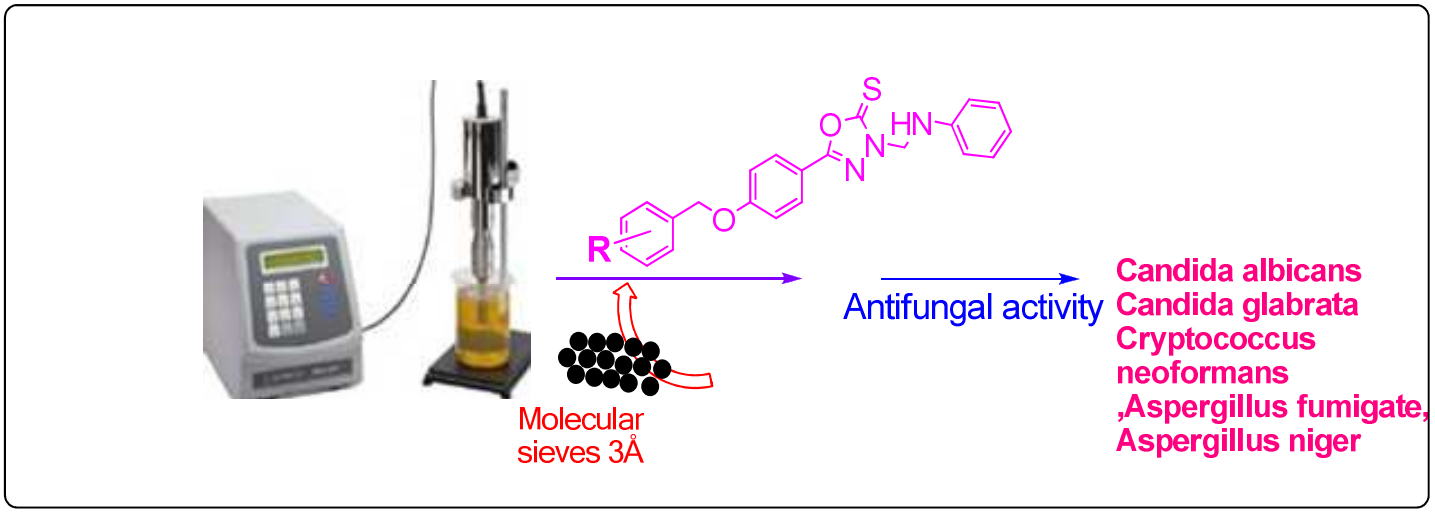
2. Maulana Azad Postgraduate & Research Centre , Aurangabad.

3. National Chemical Laboratory, Pune.

4. University of Santiago De Compostela, Lugo, Spain.

*** Email: annapratimanikalje@gmail.com**

GRAPHICAL ABSTRACT



5-(4-(Benzyloxy)substituted phenyl)-3-((phenylamino)methyl)-1,3,4-oxadiazole-2(3H)-thione as antifungal agents

"Molecular sieves and ultrasound assisted synthesis of Novel1,3,4-oxadiazole-2-thiones derivatives as potential antifungal Agents."

In the category of microorganism, fungi are considered as the special class of microbes responsible for opportunistic pathogenic infections in humans species. Due to the side effects of commercially available antifungal drugs and the emergence of new drug resistant fungal species in the past few years, has forced the researchers to search for novel and efficient antifungal drug molecules. The 1,3,4-oxadiazoles scaffold is associated with diverse biological activities. The multipurpose use of the Mannich bases in pharmaceutical chemistry provoked us to prepare a new series of 1,3,4-oxadiazole Mannich bases derivatives, as antifungal agents. Herein we report Molecular sieves and Ultrasound assisted synthesis of novel series of Mannich bases of the 5-substituted 1,3,4-oxadiazole-2-thiones by amino methylation with paraformaldehyde and substituted primary / secondary amines and their evaluation for antifungal activity .The structures of the newly synthesized compounds were determined by IR, NMR and Mass spectral study. The synthesized compounds exhibited interesting moderate to excellent antifungal activity against *Candida albicans* (NCIM 3557),*Candida albicans*(NCIM3471), *Candida glabrata*(NCIM3237), *Cryptococcus neoformans* (NCIM 3542),*Cryptococcus neoformans*(NCIM 3378),*Aspergillus fumigates*(NCIM 902), *Aspergillus niger*(NCIM 628) using Flucanazole as a standard reference drug. The synthesised compounds 6d, 6f ,6g, 6h and 6j exhibited promising antifungal activity as fungistat agents.

Key words: 1,3,4-oxadiazoles, Mannich reaction, ultrasound –assisted, Molecular sieves, antifungal activity.

CONTENTS:

- **Introduction**
- **Objective & Need of Study**
- **Materials and methods**
- **Scheme for synthesis**
- **Experimental**
- **Spectral characterization**
- ***In Vitro* Antifungal Screening**
- **Result & Discussion**
- **Conclusion**

INTRODUCTION

- The 1,3,4-oxadiazoles scaffold is associated with diverse biological activities such as antifungal[1a-b] antibacterial [2], antimycobacterial [3], antiHIV [4], anti-hepatitis B virus[5] anticancer [6] anticonvulsant [7], anti-inflammatory [8], antimalarial [9] analgesic [10] etc. 5-Substituted-1,3,4-oxadiazole-2-thiones represent an important type of compound in the field of coordination chemistry because of their potential multifunctional donor sites, viz either exocyclic sulfur or endocyclic nitrogen[11,12] and possess CNS depressant [13]and tyrosinase inhibition [14] property. The multipurpose usage of the Mannich bases in pharmaceutical chemistry [15,16] provoked us to prepare a new series of 1,3,4-oxadiazole based amino methyl derivatives . The use of ultrasound to endorse chemical reactions is called Sonochemistry. Ultrasonic-assisted organic synthesis is a green synthetic approach and it is a powerful technique towards the increase in reaction rate[17,18]. It offers the potential reaction in small time cycles, cheaper reagents and less extreme physical conditions [19,20]. It can also be considered as important tool for conservation of energy and minimization of waste as compared to the conventional techniques [21].

NEED OF STUDY

- In the category of microorganism, fungi are considered as the special class of microbes responsible for opportunistic pathogenic infections in plant, animals and humans species. Commercially available broad-spectrum antifungal drugs includes fluconazole, itraconazole, miconazole and voriconazole in which the mechanism of action is on target CYP51 which get inhibited and in turn switch off the biosynthesis of ergo sterols[22] .
- But the frequent use of these antifungal drugs in immune compromised patients who are undergoing the long term treatment of broad-spectrum antibiotics may be in cases of HIV infection, grafting surgery or anticancer therapy [23], has led to the development of resistance fungal strains.
- The emergence of new drug resistant fungal species in the past few years has force the the researchers around the world to search for novel and efficient antifungal drug molecules. [24,25,26].

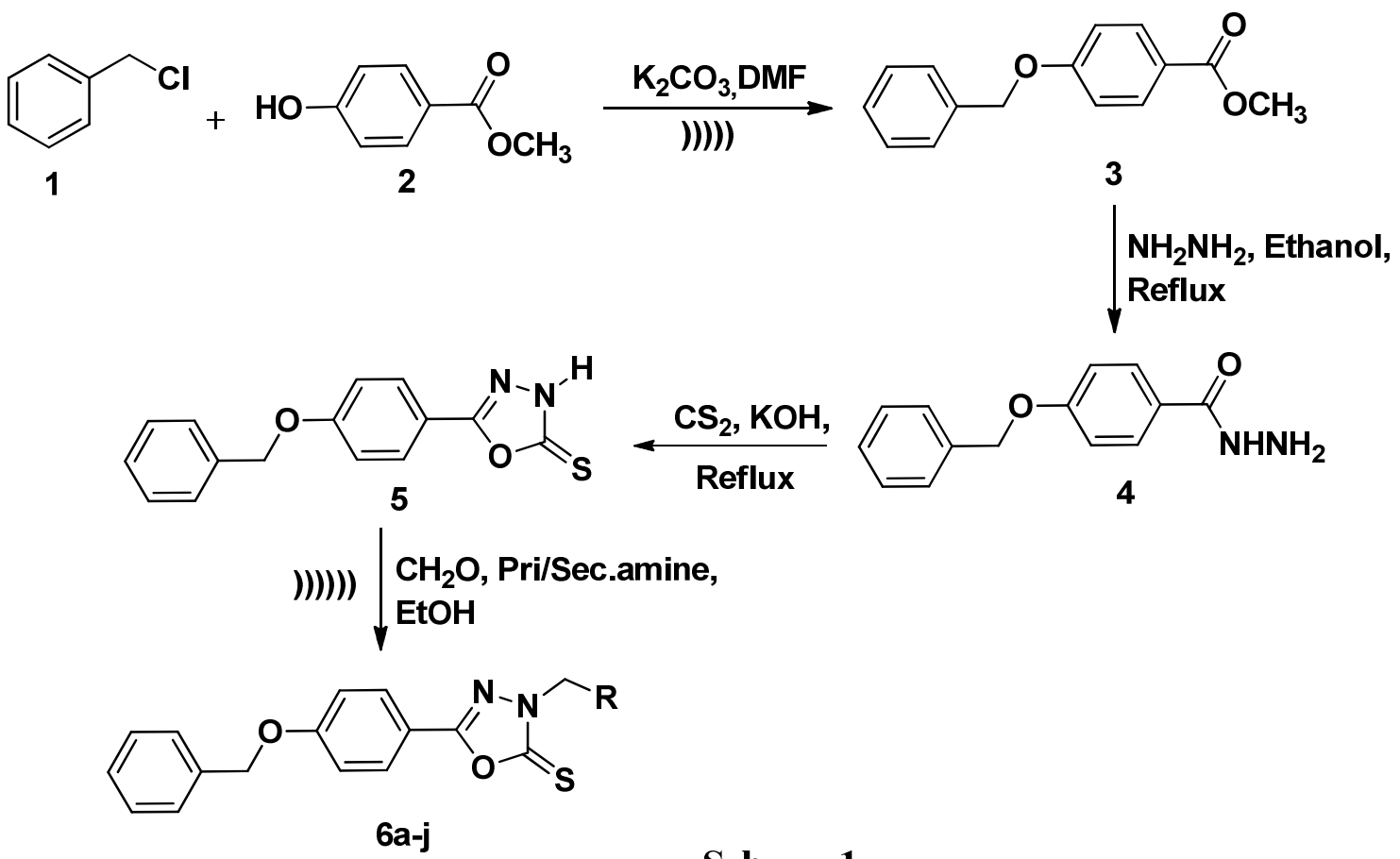
OBJECTIVE OF STUDY

- To design and synthesize the novel, 1,3,4-oxadiazole-2-thiones containing Mannich Bases by using ultrasonic processor.
- To conduct physicochemical characterization of intermediates and synthesized compounds**.**
- To confirm the structures of synthesized compounds by analytical and spectral techniques such as TLC, FT-IR, ES-MS, ^{1}H NMR and ^{13}C NMR .
- Antifungal screening of the synthesized compounds against seven selected strains of human pathogenic fungi. viz Candida albicansNCIM 3557,Candida albicansNCIM3471, Candida glabrataNCIM 3237, Cryptococcus neoformansNCIM 3542,Cryptococcus neoformansNCIM 3378,AspergillusfumigatusNCIM 902, Aspergillus niger NCIM 628.

MATERIALS AND METHODS:

All the chemicals used for synthesis were of Merck, Sigma, Research lab, Qualigens make and Himedia. The reactions were carried out by conventional method and Ultrasonic processor (P/No.VCX-500-200) for synthesis. Melting points were determined in open capillaries using melting point apparatus and are uncorrected. FTIR spectra were recorded by JASCO FTIR (PS-4000) using KBr powder technique. The ^{1}H NMR and ^{13}C NMR spectra of synthesized compounds were recorded on Bruker Avance II 400 NMR Spectrometer at 400 MHz Frequency in deuterated DMSO and $CDCl_3$ and using TMS as internal standard (chemical shift δ in ppm) at National Chemical Laboratory,Pune.

SCHEME OF SYNTHESIS



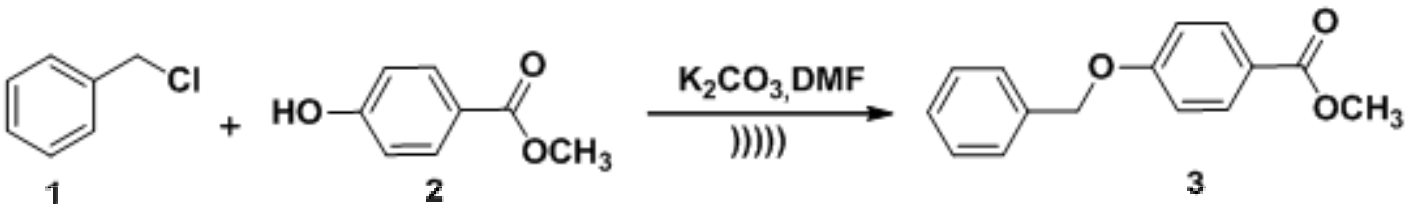
Scheme 1

5-(4-(Substituted phenyl)-3-(((4-chloro-2-nitrophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione

EXPERIMENTAL WORK & PHYSICAL CONSTANTS DATA

General procedure for the synthesis of 4-(benzyloxy)benzoate(3):

For the synthesis of4-(benzyloxy) benzoate Equimolar (0.01mol)methyl 4-hydroxybenzoate and chloromethyl benzene was taken in N,N Dimethyl Formamide (DMF) as solvent and reaction is carried out in K_2CO_3 in ultrasonic processor upto 4hr. After that, the solution was poured into ice-water. The precipitate was filtered and recrystallized from ethanol.Colour: White M.P.105^{0}C



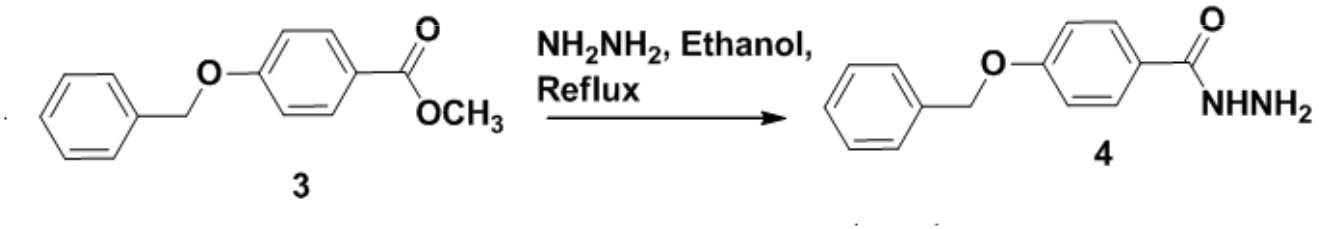
1 2 3

Compound Code	Molecular Formula	Molecular Weight	Percentage Yield (%)	Melting point	R_f value
3	$C_{15}H14O_3$	242.27	90%	105^{O}C	0.46

Solvent system chosen for R_f value determination was benzene :methanol (8:2).

GENERAL PROCEDURE FOR THE SYNTHESIS OF 4-(BENZYLOXY) BENZOHYDRAZIDE (4):

- For the synthesis of substituted benzohydrazines, a mixture of corresponding esters (20 mmol), 85% hydrazine hydrate (20 mmol) in ethanol (35 ml) was heated to reflux for 6 h. After that, the solution was poured into ice-water. The precipitate was filtered and recrystallized from ethanol. Colour: White M.P.80^{0}C



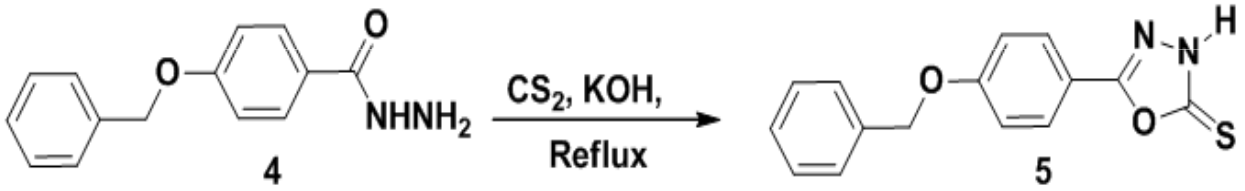
3 4

Compound Code	Molecular Formula	Molecular Weight	Percentage Yield (%)	Melting point	R_f value
4	$C15H_{14}N_2O2$	241	88%	80	0.24

Solvent system chosen for R_f value determination was benzene : methanol (8:2).

Step III: Synthesis of 1,3,4-OXADIAZOLE-2-THIONES(5)

- Then equimolar proportion of the substituted benzohydrazine(5 mmol) and potassium hydroxide (5 mmol) were dissolved in 20 mL of 95% ethanol. The mixture was allowed to stir for several minutes at room temperature and then carbon disulfide(15 mmol) was slowly added drop wise to the reaction system and the mixture was heated to reflux by using molecular sieves. The residue obtained was dissolved in water(50 mL) and diluted hydrochloric acid was added to adjust the pH values of the solution to 5-6. Then the precipitate was collected washed with water for several times and dried and recrystallized from ethanol. Colour: White M.P.160^{0}C

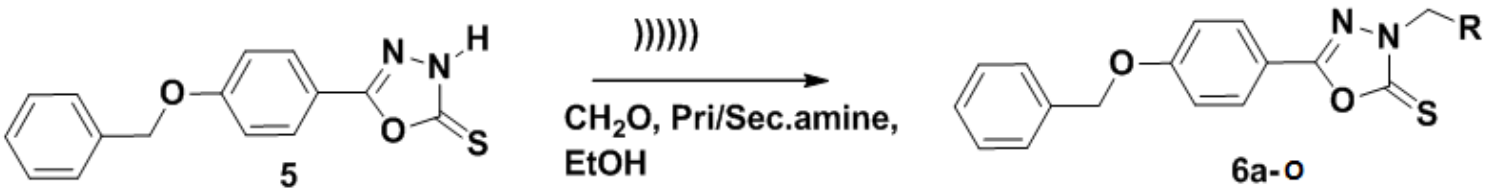


Compound Code	Molecular Formula	Molecular Weight	Percentage Yield (%)	Melting point	R_f value
5	$C_{15}H12N_2O2S1$	284	90%	160^{O}C	0.41

Solvent system chosen for R_f value determination was benzene : methanol (8:2).

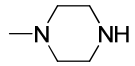
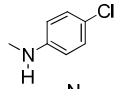
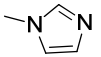
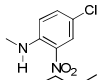
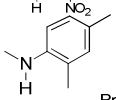
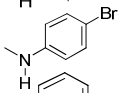
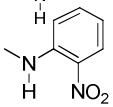
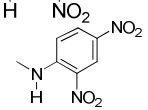
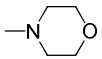
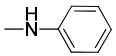
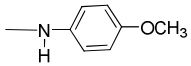
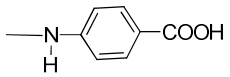
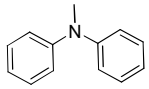
STEP IV: SYNTHESIS OF 1,3,4-OXADIAZOLE-2-THIONES MANNICH BASES:

- 1,3,4-Oxadiazole-5-thiones (10 mmol) was dissolved in methanol, then para formaldehyde (15mmol) and primary/secondary amine (10 mmol) in methanol were added with stirring. The resulting mixture was kept in ultrasonic processor for 2-4hrs . The precipitated solids were filtered, washed with water and recrystallized from methanol to yield the title compounds 6a-o (Table 1)



Solvent system chosen for R_f value determination was benzene : methanol (8:2).

Table 1.Characterization data of Mannich bases (6a-o).

Derivatives	R	(Mol. wt)	Yield (%)	m.p. (°C)	Analysis (%) Found (calculated) C	H	N
a		382.48	80	205	62.80 (62.76)	5.80 (5.74)	14.65 (14.62)
b		423.92	81	180	62.33 (62.28)	4.28 (4.22)	9.91 (9.88)
c		365.41	78	235	59.16 (59.11)	4.1 (4.10)	19.17 (19.11)
d		364.42	79	240	62.62 (62.58)	4.43 (4.40)	15.37 (15.34)
e		468.91	81	218	56.35 (56.30)	3.65 (3.61)	11.95 (11.92)
f		417.52	79	230	69.04 (69.00)	5.55 (5.51)	10.06 (10.02)
g		468.37	81	228	56.42 (56.38)	3.87 (3.84)	8.97 (8.93)
h		434.47	90	220	60.82 (60.78)	4.18 (4.13)	12.90 (12.86)
i		479.47	82	145	55.11 (55.08)	3.57 (3.52)	14.61 (14.57)
j		383.46	85	200	62.64 (62.61)	5.52 (5.48)	10.96 (10.92)
k		389.47	80	80	67.84 (67.79)	4.92 (4.88)	10.79 (10.74)
l		419.13	81	100	65.85 (65.81)	5.05 (5.00)	10.02 (10.00)
m		433.48	79	140	63.73 (63.67)	4.42 (4.39)	9.69 (9.62)
n		327.40	78	110	62.36 (62.31)	5.23 (5.19)	2.83 (2.79)
o		465.57	79	115	72.23 (72.20)	4.98 (4.92)	9.03 (9.00)

ANTIFUNGAL SCREENING

In vitro antifungal susceptibility testing were performed by broth micro dilution method according to the Clinical and Laboratory Standards Institute (CLSI) to find out IC_{50} concentration and minimum inhibitory concentration (MIC_{90}) of the compounds. The activities of compounds were checked against human pathogens *Candida albicans* NCIM 3557,*Candida albicans* NCIM3471, *Candida glabrata* NCIM 3237, *Cryptococcus neoformans* NCIM 3542,*Cryptococcus neoformans* NCIM 3378,*Aspergillus fumigatus* NCIM 902, *Aspergillus niger* NCIM 628.

PROTOCOL FOR ANTIFUNGAL ACTIVITY

- Stock of each compounds were prepared in DMSO at concentration of 12800 µg/ml. Stocks were serially diluted two fold in micro titer plate and 4µl of this is used for assay to get a final concentration in the range of 256-2 µg/ml.
- Spores of the filamentous fungi (~$2x10^4$ spores/ml) and yeast cells freshly grown in YPG broth in logarithmic phase (~$2x10^3$cfu/ml) were suspended in the RPMI 1640 medium and 196 µl from these were inoculated in the wells of the plate.
- The microtitre plate were incubated for 24-48hr.
- Growth was determined by visual observation and measuring absorbance at 600 nm using micro titre plate reader.
- The IC_{50} was defined as the concentration exhibiting 50% inhibition of the growth as compared to the growth of control. Whereas MIC_{90} was the concentration causing>90% inhibition of the growth as compared to the growth of control.

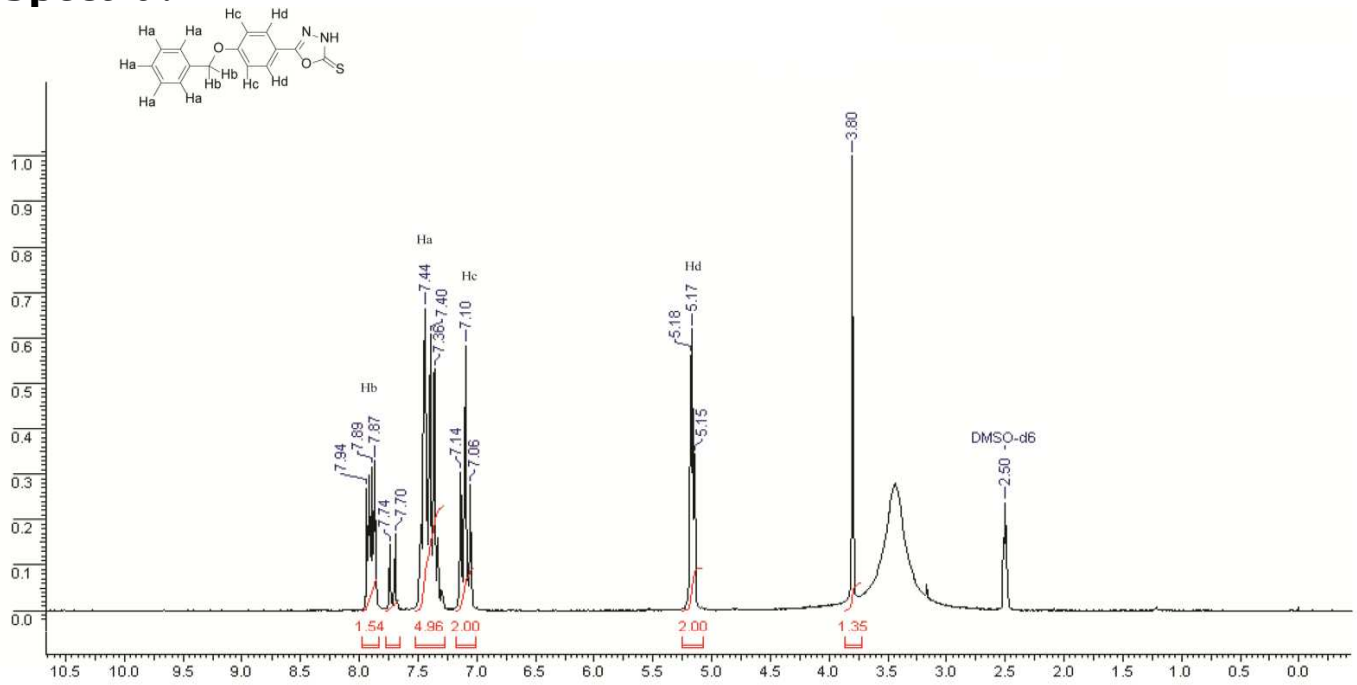
Table 2: Representation of IC50 Antifungal screening of 6(a-o)

Entry	*Candida albicans* (NCIM 3557)	*Candida albicans*(NCIM 3471)	*Candida glabrata*(NCIM 3237)	*Cryptococcus neoformans* (NCIM 3542)	*Cryptococcus neoformans* (NCIM 3378)	*Aspergillus fumigatus*(NCIM 902)	*Aspergillus niger.* (NCIM 628)
6a	64	49.6	64	64	64	>256	**27.7**
6b	128	67.12	59.6	77.5	>128	**24.2**	**24.1**
6c	21.6	108	53.6	108.9	196.5	**11.2**	93
6d	68	159	65.6	128	>128	**6**	130
6e	54.4	44.6	**24.5**	64	**14.21**	**25.8**	**47**
6f	**16**	84.9	**5.4**	>256	90	-	>256
6g	62	107.6	256	76.8	94.7	**4**	>256
6h	55.3	57.4	64	47.6	66.6	**16**	**18.61**
6i	23.3	80.6	40.3	256	93.5	**24.6**	>256
6j	38.4	31.5	56.6	192	39	**8**	128
6k	150	53.3	36.2	64.2	146.6	35.1	**22.9**
6l	115	95.3	42.5	49.7	145.8	81	73.2
6m	91.5	49.7	78.6	62.5	54.6	87.4	84.8
6n	30.3	43	48.8	47.5	105.6	81.5	56.9
6o	95	**28.1**	50.7	**42.7**	156.5	76.1	70.5
Fluconazole	0.12	0.11	9.4	16	4	64	46

TABLE 3:REPRESENTATION of MIC of Antifungal screening of 6(a-o)

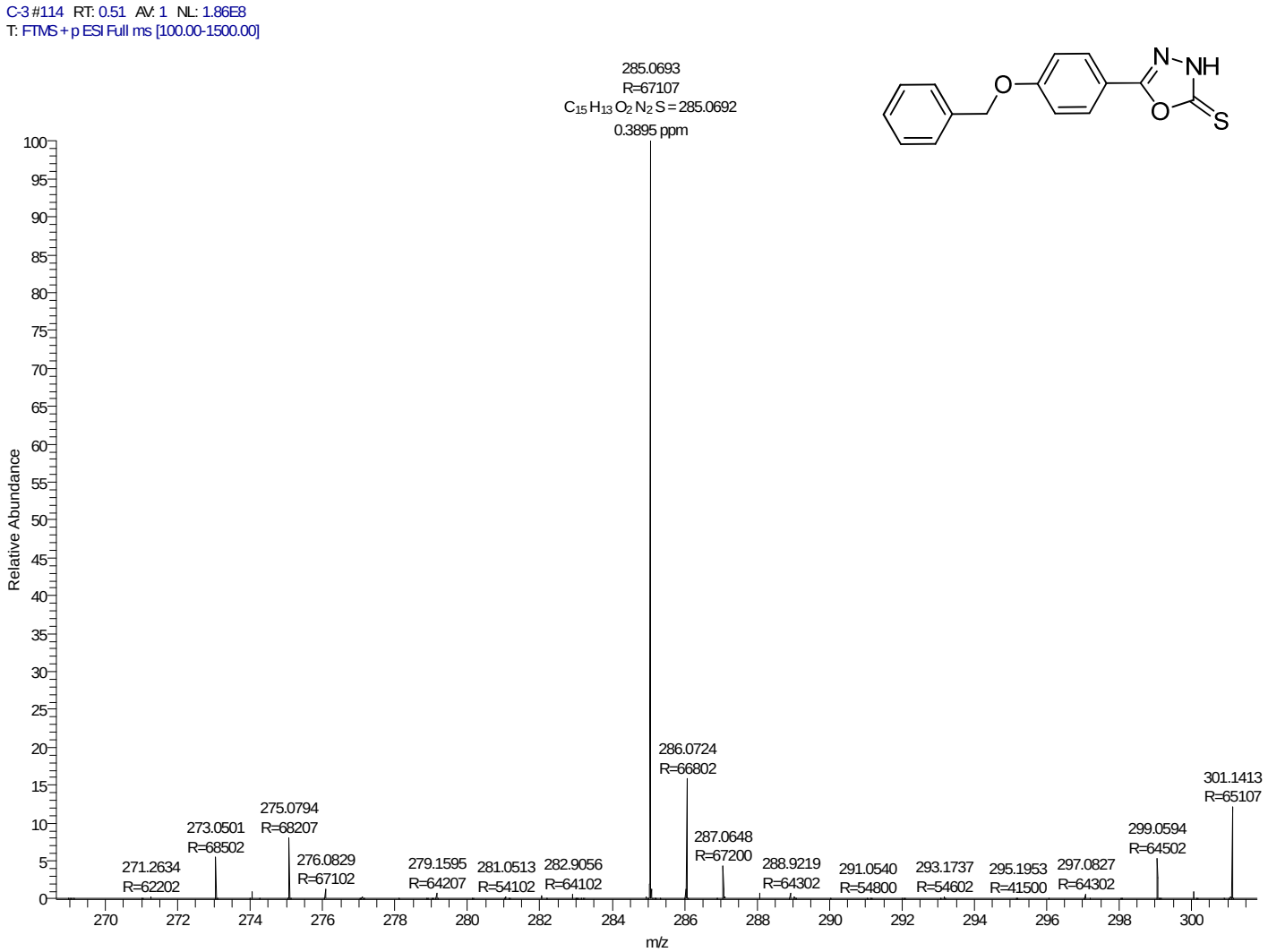
Entry	*Candida albicans*(NCIM 3557)	*Candida albicans*(NCIM 3471)	*Candida glabrata*(NCIM 3237)	*Cryptococcus neoformans* (NCIM 3542)	*Cryptococcus neoformans* (NCIM 3378)	*Aspergillusfumigatus*(NCIM 902)	*Aspergillusniger.* (NCIM 628)
6a	>256	>64	64	>256	>256	>256	>**64**
6b	>256	128	128	>256	>256	**128**	>256
6c	128	128	128	>256	>256	**128**	>256
6d	>256	>256	>128	>256	>256	256	>256
6e	64	64	64	256	>256	**128**	256
6f	64	256	**8**	>256	>256	256	256
6g	128	256	256	256	>256	256	256
6h	128	>256	256	>256	>256	256	>256
6i	**32**	128	64	256	256	256	**128**
6j	128	256	256	256	>256	>256	256
6k	>256	>256	>256	>256	>256	>256	>256
6l	>256	>256	>256	>256	>256	>256	>256
6m	>256	>256	>256	>256	>256	>256	>256
6n	>256	>256	>256	>256	>256	>256	>256
6o	128	**32**	128	**64**	>256	**128**	**128**
Fluconazole	**0.25**	**0.25**	**16**	**32**	**8**	**>256**	**>256**

^{1}H Spectra:



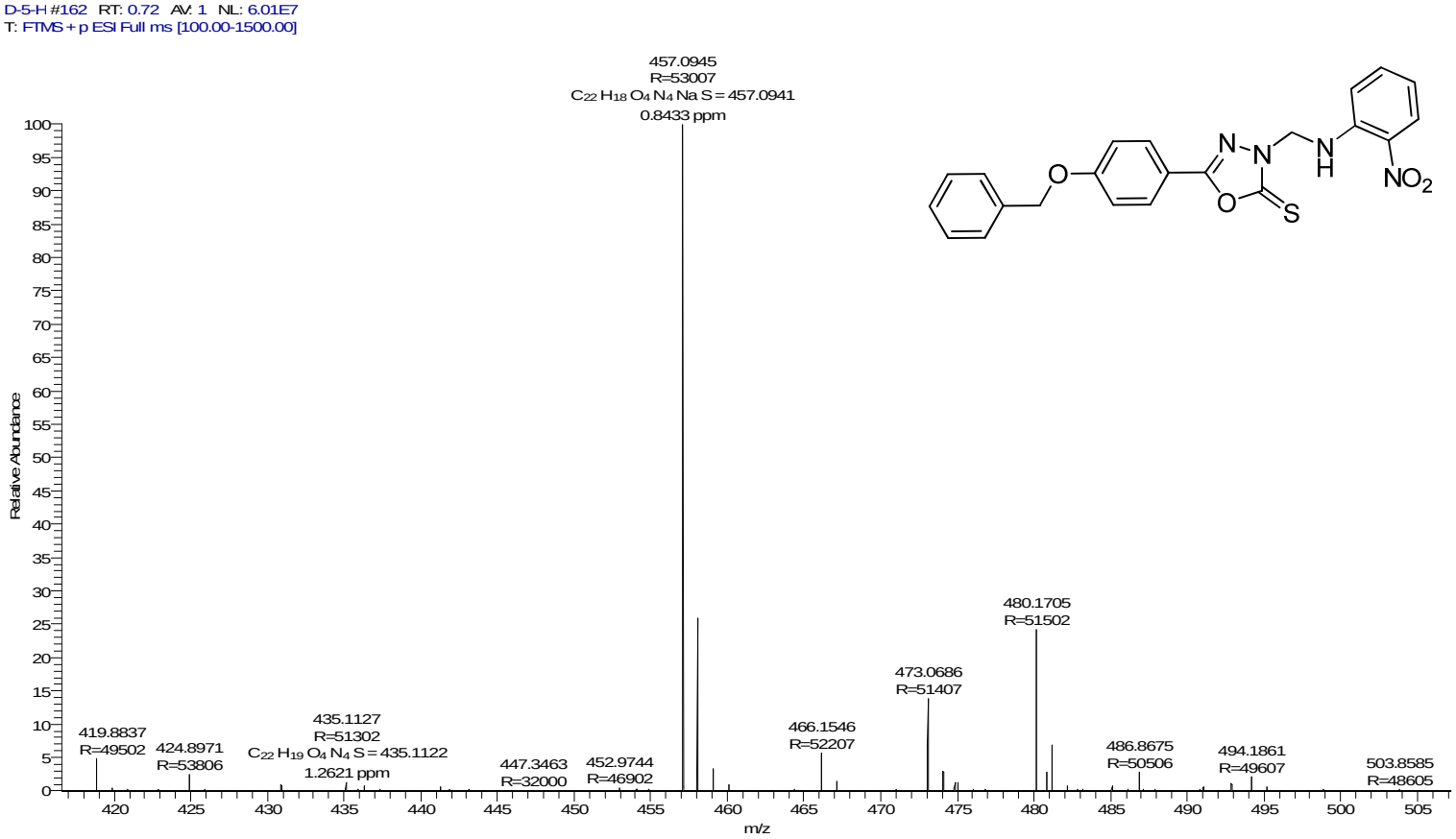
Sr. No.	δ Values (ppm)	Multiplicity	No. of proton	Group
1	3.8	s	1H	N-H
2	5.1-5.2	m	2H	Hd
3	7.0-7.1	m	2H	Hc
4	7.3-7.4	m	5H	Phenyl ring (Ha)
5	7.8-7.9	s	2H	-CH_2 (Hb)

Mass Spectra:



Sr. No.	Fragmentation	m/e
1	M+1	285
2	M+2	286

Mass Spectra:



Mass fragmentation

Sr. No.	Fragmentation	m/e
1	M+23(Na)	457
2	M+1(Na)	458
3	M+2(Na)	459

RESULTS AND DISCUSSION:

- Among the synthesised compounds **6g**(4µg/ml),**6d**(6µg/ml),**6j**(8µg/ml),**6c**(11.2µg/ml),**6h**(16µg/ml),**6b**(24.2µg/ml),**6i**(24.6µg/ml),6e(25.8µg/ml) & 6k(35.1µg/ml) give **excellent** activity against the strain *Aspergillus fumigatus* (NCIM 902) for which the standard drug fluconazole required is (64 µg/ml).
- Similarly compounds **6h**(18.61µg/ml),6k(22.9µg/ml),**6b**(24.1µg/ml) & 6a(27.7µg/ml) gives **excellent** activity against the *Aspergillus niger*.(NCIM 628),whereas 6e(47µg/ml) give good activity against the same strain, the standard drug fluconazole required is (46 µg/ml).
- Compound 6f (5.4 µg/ml) gives **excellent** activity against *Candida glabrata*(NCIM 3237) and 6e(24.5µg/ml)show **good** activity, the standard drug fluconazole required is (9.4 µg/ml).
- Other synthesized compound show good to moderates activity for the remaining strain of fungi.

CONCLUSION:

Fifteen novel derivatives of **5-(4-(benzyloxy)substituted phenyl)-3-((phenylamino)methyl)-1,3,4-oxadiazole-2(3H)-thione** were successfully synthesized under ultrasound irradiation giving better yield of 75-95% and in shorter duration of 2-4 hrs in contrast to conventional reactions which requires refluxing of 10-12 hrs. . The structures of all the compounds were confirmed by recording their ^{1}H NMR, ^{13}C NMR, Mass and IR spectra. All the newly synthesized compounds were screened for their in vitro antifungal properties. The compounds were found to be fungi static. Among the screened samples, nearly nine derivatives exhibited excellent antifungal activity against ***Aspergillus fumigatus*** (NCIM 902), fluconazole was used as standard drug, (64 µg/ml).The excellent antifungal activity exhibited by the following compounds may be due to the presence of various electron withdrawing groups. All the mentioned derivatives give excellent activity, such as **6g**(4µg/ml) having bromine at para position of phenyl act as strong withdrawing groups is found to be the most potent, **6d**(6µg/ml) having imidazole, **6j**(8µg/ml) morpholine, **6c**(11.2µg/ml) having triazole, **6h**(16µg/ml) having nitro at para position of phenyl and **6b(**24.2µg/ml) having para substituted chlorophenyl, **6i**(24.6µg/ml) show two nitro phenyl group at ortho and para position, **6e**(25.8µg/ml) ortho nitro and para chloro phenyl and **6k**(35.1µg/ml) having un substituted phenyl.

- Other compounds which exhibited excellent antifungal activity **6h**(18.61µg/ml),6k(22.9µg/ml),**6b**(24.1µg/ml) & **6a(**27.7µg/ml) having piperazine as a substituent, **6e**(47µg/ml) against ***Aspergillus niger***.(NCIM 628) and fluconazole, standard drug required is (46 µg/ml). Some compounds exhibited excellent to good activity against ***Candida globrata***(NCIM3237) when compared with the same standard drug fluconazole (9.4 µg/ml) such as **6f** (5.4 µg/ml) ortho and para di substituted toluyl; **6e**(24.5µg/ml)shows good activity .
- With these excellent result here we can conclude that **5-(4-(benzyloxy)substituted phenyl)-3-((phenylamino)methyl)-1,3,4-oxadiazole- 2(3H)-thione** derivatives can act as a potent scaffold to develop newer drugs possessing antifungal activity. The final derivatives possessing electron withdrawing groups on phenyl ring at position 3 of nitrogen of 1,3,4-Oxadiazole-2-thiones ring are acting as more potent compounds and exhibit excellent antifungal activity .

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