An Efficient Combinatorial Approach for Beta-Lactam Antibiotics with Novel Adjuvants against Gram-Negative Organisms to Combat Multi-Drug Resistance

Chandresh Pareek*[1], Dushyant Pareek[2], Narendra Nirwan[3], Ajay Jain[4]

[1]Associate Prof. Microbiology Research Lab, S.D. Govt. College Beawar, Rajasthan, INDIA
[2]Associate Prof. Microbiology Research Lab, S.D. Govt. College Beawar, Rajasthan, INDIA
[3]Assistant Prof. Microbiology Research Lab, S.D. Govt. College Beawar, Rajasthan, INDIA
[4]Associate Prof. JLN-Medical College-Ajmer INDIA

Abstract

Anti-Microbial Resistance [AMR] is progressively grave menace to global wellness. New techniques and developments are almost none to least to overcome this. Herein we describe recent development toward combinatorial in-vitro therapy for the treatment of multi-drug resistant bacterial infections and we have approached to this by eliminating the overuse of antibiotics as well as by the use of an 'antibiotic adjuvants' in combination with an antibiotic. These attempts include the development of adjuvants that either directly target resistance mechanisms such as the inhibition of beta-lactamase enzymes or indirectly target resistance to two-component systems. To this end, we utilized and developed the anti-resistance adjuvants that cause multi-drug resistant species to become susceptible to clinical antibiotics that are no longer effective. From the initial in-vitro screening, we developed new imidazole adjuvants that decreased the effective concentration of popular beta lactam antibiotics against *Pseudomonas aeruginosa and E. Coli* towards a clinical multi-drug resistant. For this we calculated MIC [Minimum Inhibitory Concentration] exclusively for selected drugs and employed it with adjuvants.

Keyword: Anti-microbial Resistance; GNB, β-lactams; substituted imidazoles; MIC

1. Introduction:

Although all bacteria have an inner cell membrane, gram-negative bacteria have a unique outer membrane. This outer membrane excludes certain drugs and antibiotics from penetrating the cell, partially accounting for why gram-negative bacteria [GNB] are generally more resistant to antibiotics than are gram-positive bacteria. Production of beta-lactamases, the enzymes that resist to beta-lactam antibiotics, is the most widespread and threatening mechanism of antibiotic resistance [1]. Both ampicillin and amoxicillin are β-lactam antibiotics and susceptible to β-lactamase also. Amoxicillin has a spectrum of activity similar to that of ampicillin but is better absorbed; because it inhibits β-lactamases therefore clavulanic acid is added to prepare amoxicillin combination [2] whereas the classical Penicillin-G shows a relatively poor antibiotic activity against GNB such as *E. coli. Pseudomonas aeruginosa* is another kind of GNB which is a notoriously difficult organism to control with antibiotics or disinfectants. That is why two agents from different classes should be used when the risk of antibiotic resistance is high [3]. So on the whole gradually and progressively consumption of antibiotics or their demand is increasing as they are attending to be resistant. No alternate therapy is yet in state to replace it. Since fewer new antibiotics targeting GNB are in development and organisms that

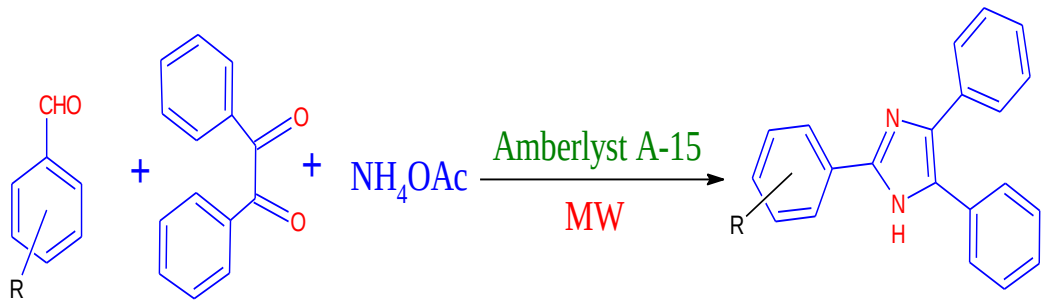
resist all or most clinically useful antimicrobials are already being isolated, drug-resistant infections due to GNB represent a significant health care concern for the future [4]. WHO has published a Global Priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics. It addresses the urgent need for research efforts for Gram negative bacteria-Multi drug resistance [5]. To get the better of this problem, the use of an 'antibiotic adjuvant' in combination with an antibiotic is now being developed. This approach enables us to sustain the lifespan of these life-saving drugs. Taken up together, it appears clear that it is essential to explore not only for more effective anti-infective drugs but also to formulate novel chemical entities with new mechanisms of action. In contrast, greater success has been attained with those approaches that minimize the strike of resistance to antibiotics by the use of an 'antibiotic adjuvants' in combination with an antibiotic.

Antibiotic adjuvants: These compounds are also termed as 'resistance breakers' or 'antibiotic potentiators' [6-9] and they have little or no antibiotic activity but co-administered with the antibiotic they either (i) block the main bacterial resistance mechanisms or (ii) enhance the antimicrobial action of the drug. From the drug discovery point of view, this combined drug therapy has the advantage that it is not necessary to spend effort in the challenging and expensive identification of new targets that are essential for bacterial survival. To serve this purpose Imidazole substituents (Fig-2) may work as potential adjuvants.

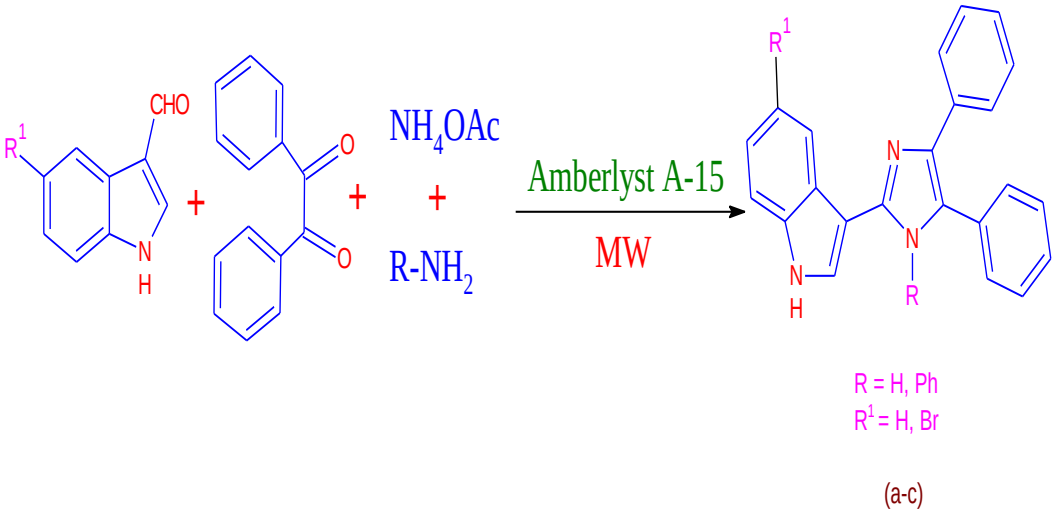
Imidazole is an important class of heterocycles being the core fragment of different natural products and biological systems. Compounds containing imidazole moiety have many pharmacological properties and play important roles in biochemical processes [10]. Omeprazole, Pimobendan, Losarton, Olmesartan, Eprosartan and Triphenagrel are some of the leading drugs in the market with diverse functionality. Tri-arylimidazoles are used in photography as photosensitive compounds. In addition, the imidazole derivatives have many biological activities, such as herbicidal, analgesic, fungicidal, anti-inflammatory and antithrombotic activities [11-14].

In our context, modified approach for the previously reported novel bio active imidazole [15] adjuvants was also planned using 'Amberlyst A-15' Catalyst [16] as:

***Scheme** I*



Scheme II

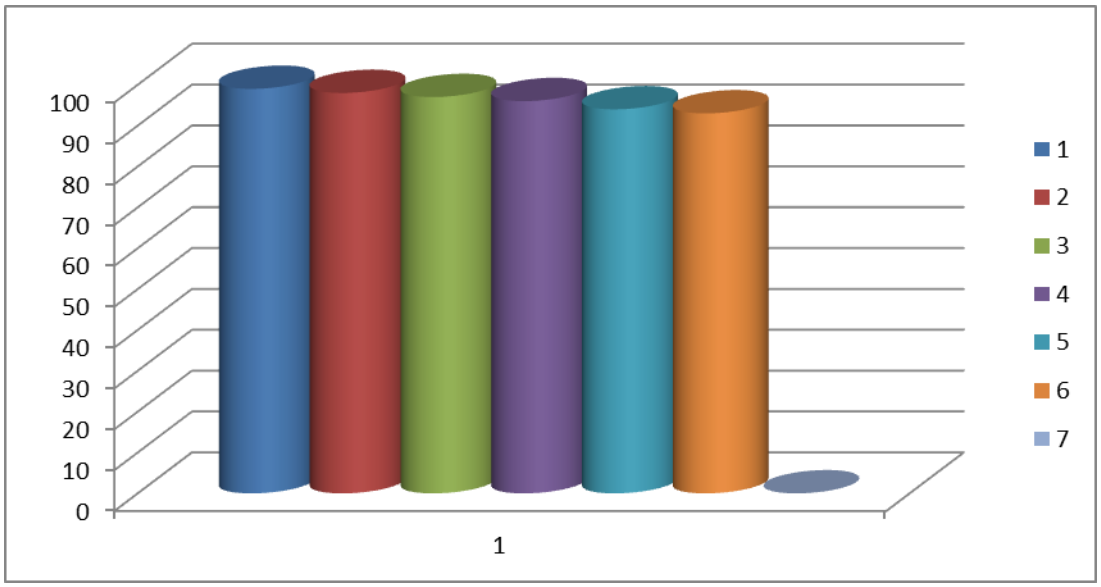


2. Methods

2.1 Brief Procedure and Reusability of Catalyst

Mixture of benzil (2 mmol), aldehyde (2 mmol), NH_4OAc (2 mmol) and Amberlyst A-15 (140 mg) was brought for one pot synthesis and irradiated with BP110 Laboratory Grade Microwave. The progress of reaction and purity of yield was monitored in regular interval by TLC using petroleum ether and ethyl acetate (9:1). After completion of the reaction the dichloromethane was added and the solid Amberlyst-A-15 was filtered and dried at 80°C and used for its reusability (Fig-1) [17]. The organic layer was extracted with H_2O and dried by Na_2SO_4. Purity of final imidazole compounds was further correlated with reference spectral and other data. After finding in agreement with data, the selected compounds (Tab-1) were brought forward for biological screenings.

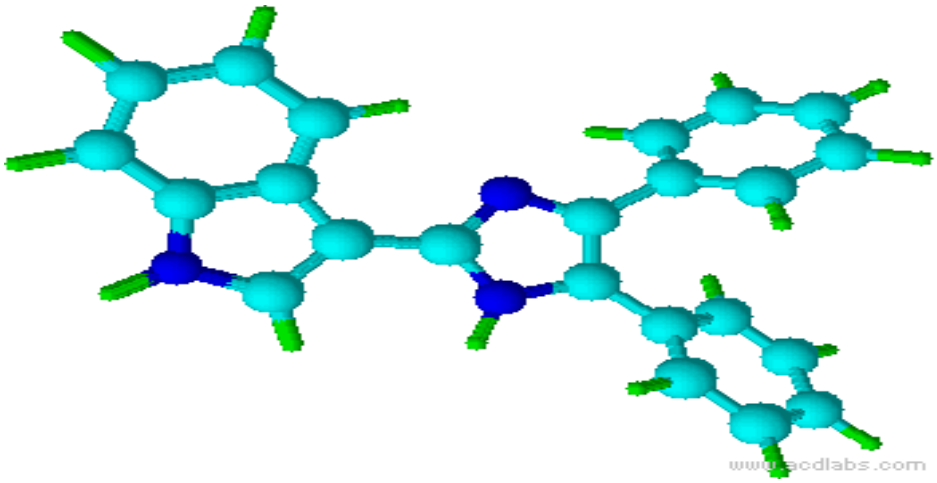
Figure-1. The Reusability of the catalyst for Modal reaction (f)



Tabel-1 Imidazole Substituents

No.	R_1	R	% Yield	Reaction Time (min)	mp °C	mw	Molecular Formula
a	-	H	98	11	271-272	296.365	$C_{21}H_{16}N_2$
b	-	p-Br	95	13	248-252	375.258	$C_{21}H_{15}BrN_2$
c	-	p-NO_2	94	15	241-242	341.359	$C_{21}H_{15}N_3O_2$
d	-	p-Cl	96	14	262-264	330.807	$C_{21}H_{15}ClN_2$
e	-	p-MeO	93	16	229-231	326.386	$C_{23}H_{18}N_2O$
f	H	H	97	12	158-161	335.402	$C_{23}H_{17}N_3$
g	Br	H	95	14	237-239	414.29724	$C_{23}H_{16}BrN_3$
h	H	Ph	94	17	233-235	411.49714	$C_{29}H_{21}N_3$

Figure-2 Substituted indolylimidazole



Source: (acdlabs)

2.2 Biological screening

Our primary aim is to evaluate biological activities of all the synthesized imidazole compounds employing combinatorial approach with selected reference antibiotics.

2.2.1 Preliminary screening for antibacterial activity:

In the study of antibacterial activity, the test organisms used were: Gram negative bacillus *E. coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and 530 strains of Gram negative bacteria including 120 clinical isolates collected from the Microbiology lab of the All India Institute of Medical Sciences [AIIMS] and JLN Medical College-Ajmer INDIA.

The antibacterial activities of individual reference substances and of two-drug combinations were evaluated for 530 strains of Gram negative bacteria including 120 clinical isolates. The test was performed by disc diffusion assay as per CLSI guideline M02 [18]. The sample [a-h] was dissolved in methanol 100 µg mL^{-1} concentration for bacterial studies. The nutrient agar plates containing an inoculum size of 106 cfu mL^{-1} for bacteria was used. Previously prepared impregnated discs (whatman's filter paper of 6 mm in diameter) in sample solution with control solution of strains were placed aseptically on sensitivity plates, Cefotaxime-10, Penicillin-10, Ampicillin-25 and Amoxicillin-25 (µg) were used as standard antibacterial antibiotics. Plates were incubated at 37^0C for 24 hours for bacterial growth. Sensitivity was recorded by measuring the clean zone of inhibition in millimetres on agar surface around the disc (Tab 2-3). The process was repeated in triplicate for accuracy.

2.2.2 Zone of inhibition after mixing of compounds with reference antibiotics

All the imidazole compounds [a-h] were again checked for their biological sensitivity against similar test organisms but after mixing (1:1) with reference sample as adjuvants in same environment at 37^0C for 24 hours.

Table 2 *- Zone of Inhibition against clinical isolates in mm*

adjuvants (added with antibiotics) 1:1	Diameter of zone of inhibition (mm) against E.Coli			
	Cefotaxime-10	*Amoxicillin-25*	*Penicillin-G*	*Ampicillin-25*
a +	18 ± 1	--	--	15 ± 2
b +	16 ± 2	--	--	15 ± 1
c +	42 ± 2 ++	26 ± 4	12 ± 4 ++	32 ± 6 ++
d +	38 ± 5 ++	24 ± 5	12 ± 5 ++	21 ± 2
e +	16 ± 5	--	--	15 ± 1
f +	19 ± 6	--	--	
g +	38 ± 5 ++	32 ± 8 ++	15 ± 5 ++	38 ± 5 ++
h +	35 ± 5 +	30 ± 2	12 ± 5 ++	38 ± 5 ++
Ref against ATCC25922	19-35	19-25	--	20-30
Against clinical isolates without adjuvants	12 ± 5	--	--	10 ± 2

--Not Noticeable, ++ shows significant activity

Table 3 *- Zone of Inhibition against clinical isolates in mm*

adjuvants (added with antibiotics) 1:1	Diameter of zone of inhibition (mm) against P.aeruginosa			
	Cefotaxime-10	*Amoxicillin-25*	*Penicillin-G*	*Ampicillin-25*
a +	17 ± 1	--	--	--
b +	15 ± 1	--	--	--
c +	27 ± 5 ++	10 ± 5	12 ± 5	18 ± 5
d +	26 ± 3 ++	08 ± 4	10 ± 5	16 ± 4
e +	14 ± 4	--	--	--
f +	15 ± 2	--	--	--
g +	38 ± 5 +++	12 ± 4	17 ± 5	18 ± 5
h +	36 ± 5	08 ± 5	08 ± 5 +	10 ± 5
Ref against ATCC27853	18-22	--	--	--
Against clinical isolates without adjuvants	12 ± 3	--	--	--

--Not Noticeable, ++ shows significant activity

2.2.3. Determination of minimum inhibitory concentration (MIC) Breakpoint:

The MIC was determined by two fold dilution method as per CLSI, M07 [19] for each of the test organisms in triplicates. To 0.5 mL of varying concentrations of sample (0 - 100 µg mL^{-1} for bacterial strains), 1 mL of nutrient broth was added. Then, a loopful of test organism (previously diluted to 0.5 McFarland turbidity standards for Bacterial isolates was introduced to the tubes. The procedure was repeated on the test organisms using standard antibiotics. A tube (containing nutrient broth seeded with test organisms) served as a control. The tubes containing bacterial cultures were then incubated at 37^0C for 24 hours for bacteria. After incubation the tubes were examined for microbial growth. MIC breakpoint values were checked for each drug individually and in combination (1:1) with adjuvants. [Tab. 4-5]

***Table 4**-Results of Minimum Inhibitory Concentration 'MIC' Breakpoint in $\mu g\ mL^{-1}$ against E. coli*

Adjuvants (added with antibiotics) 1:1	*Cefotaxime* MIC S≤	R>	*Amoxicillin* MIC S≤	R>	*PenicilliG* MIC S≤	R>	*Ampicillin* MIC S≤	R>
c +	.004	.50	8	32	128	128	4	8
d +	.004	.50	8	32	128	128	8	8
g +	.008	.50	2	32	64	128	2	8
h +	.008	.50	2	32	--	--	2	8
Ref against ATCC25922	.016	.50	16	32	IE	IE	32	64
without adjuvants	.50	2	R	R	R	R	R	R

-*.....Not Noticeable, Range above is for all isolates*

***Table 5**-Results of Minimum Inhibitory Concentration 'MIC' Breakpoint in $\mu g\ mL^{-1}$against P.aeruginosa*

Adjuvants (added with antibiotics) 1:1	*Cefotaxime* MIC S≤	R>	*Amoxicillin* MIC S≤	R>	*PenicilliG* MIC S≤	R>	*Ampicillin* MIC S≤	R>
c +	1	32	R	R	R	R	R	R
d +	1	32	R	R	R	R	R	R
g +	.25	32	128	512	R	R	128	512
h +	.50	32	R	R	R	R	R	R
Ref against ATCC7853	2	32	R	R	R	R	R	R
without adjuvants	64	128	R	R	R	R	R	R

-*.....Not Noticeable, Range above is for all isolates*

2.3 Results and Discussion

In this context we prepared previously reported eight imidazole substituents using 'Amberlyst A-15' catalyst to achieve maximum yield and maintain purity in nontoxic environment which is all-important for biological screening.

The in-vitro activity of all the four antibiotics was determined against 530 isolates of Pseudomonas *aeruginosa* and *E. coli* separately. We tested the combination of imidazole adjuvants (prepared using Amberlyst A-15 catalyst, Table-1) with a standard antibiotic like penicillin, ampicillin, amoxicillin and cefotaxime i.e. drugs, which differ in their mode of action.

The preliminary antibacterial activities for all the combinations have been shown in Table 2-3. In the terms of the zone of inhibition, out of all the eight selected imidazole derivatives, the maximum sensitivity was shown by "d, e, g and h" compounds when used as adjuvants with reference antibiotics. The sensitivity of

antibiotics increased up to prominent extent. Although except cefotaxime remaining β-lactams used were found to be resistance against almost all the clinical isolates. Surprisingly as we tested imidazole adjuvant 'c' against E. coli in combination of antibiotics, the susceptibility achieved as >42 mm from >19mm for cefotaxime, >32mm from >19mm for amoxicillin with 'g' and >38mm against 20mm for 'g' and 'h' with ampicillin respectively. Thus noteworthy susceptibilities were observed by c,d,g and h against E. coli whereas penicillin didn't show much improvement over rest of the combinations. This is further affirmed by their respective MIC values also. The breakpoint for cefotaxime against *E.coli* [Tab-4] with 'c' and 'd' are S ≤0.004 / R >.5 and S ≤.008 / R >.50 respectively. Both amoxicillin and ampicillin show their breakpoints S ≤2 / R >32 and S ≤2 / R >8 respectively with combinations to 'g and 'h' adjuvants.

P. aeruginosa is considered as resistant to most β-lactams but when we applied our conjugation therapy so again significant results were obtained for cefotaxime as values of zone of inhibition accomplished to >38 from >18mm for adjuvant 'g', rest of all combinations showed relatively poor sensitivity although better than reference. These outcomes are again supported by their MIC values. The breakpoint for cefotaxime against *P.aeruginosa* with 'c' and 'd' are S ≤1 / R >32 and for 'g' and 'h' are S ≤0.25 / R >32 respectively. Therefore, a substantial lowered value in MIC is recorded. Significant activity of 'g' adjuvant was depicted against both *E. coli* and *P. aeruginosa* whereas 'c' also showed excellent susceptibility when added with cefotaxime and ampicillin.

3. Conclusion

The in-vitro results for combination of β-lactams (cefotaxim, amoxicillin, ampicillin and penicillin) are presented in Tab. 2-5, where we have presented an efficient combinatorial approach of antibiotics with imidazole derivatives and significant antimicrobial susceptibility is observed against *E. coli* and *P. aeruginosa*. Thus, those β-lactams supposed to be resistant against Gram-negative bacteria became susceptible again when used in combinations with selected imidazole. Moreover, this combinatorial approach of antibiotics with adjuvants may lead to the development of new and vital antimicrobials against *E. coli* and *P. aeruginosa*, although further actions are needed to characterize the possible interaction mechanism between these antibiotics with adjuvants. Therefore, these active compounds can be exploited as antibacterial agents for the production of new drugs but routine use of combinations of antibiotics should, however, be exercised with caution after prior *in-vivo* trials.

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References

[1] Zeng X, Lin (2013*)* Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria, *J Front Microbiol* vol-4: pp 128
[2] David J. Maggs, Slatter's Fundamentals of Veterinary Ophthalmology, 4th Edition (2008) *Chapter 3 – Ocular Pharmacology and Therapeutics pp 33 – 61.* Elsevier Inc.
[3] Hancock RE, (1998) Resistance mechanisms in Pseudomonas aeruginosa and other non-fermentative gram-negative bacteria, *Clin Infect Dis*;1(suppl): S-93.
[4] Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ, (2015) Molecular mechanisms of antibiotic resistance, *Nat Rev Microbiol* vol 13(1), pp 42–51.
[5] World Health Organization (WHO), (2017) *Global priority list of antibiotics-resistance bacteria to guide research, discovery and development of new antibiotics* Feb 27, 2017.
[6] E.E. Gill, O.L.Franco, R.E.W. Hancock, (2015), Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens *Chem Biol Drug Des,* 85 pp. 56-78
[7] C.G.Bello, (2017), Antibiotic Adjuvants A strategy to unlock bacterial resistance to antibiotics, *Bioorganic & Medicinal chemistry Letters, Elsevier,* vol-27(18) pp 4221-4228
[8] M.A. Farha, E.D. Brown**,** (2013), Discovery of antibiotic adjuvants **(**2013**),** *Nat Biotech,* 31(2) p. 120-2
[9] D. Brown, (2015) Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? *Nat Rev Drug Discov.*, 14, pp. 821-832,
[10] Sundberg R J, Martin R B, (1974), Interactions of histidine and other imidazole derivatives with transition metal ions in chemical and biological systems, ***Chem. Rev*** 74 (4)**,** 471-517
[11] Boiani M., Gonzalez M., (2005), Imidazole and benzimidazole derivatives as chemotherapeutic agents *Mini Rev Med Chem*., 5(4), 409-424
[12] Chen J., Wang Z., Lu Y., Dalton J.T., Millera D.D., Li W., (2008), Synthesis and antiproliferative activity of imidazole and imidazoline analogs for melanoma, *Biorg. Med. Chem. Lett*., 18, 3183-3187
[13] N. Rani, A. Sharma, G. Gupta, R. Singh, (2013), Imidazoles: as potential antifungal agents : A Review, *Mini Reviews in Medicinal Chemistry*., 13(11) 1626-1655
[14] lmasirad A, Mousavi Z, Tajik M, Assarzadeh MJ, Shafiee A, (2014), Synthesis, analgesic and anti-inflammatory activities of new methyl-imidazolyl-1,3,4-oxadiazoles and 1,24-triazoles, *DARU J. Pharmaceutical sciences.* 22(1), 22
[15] Pareek C.; Nirwan N.; (2016) Synthesis of Bioactive Molecules And Their Biological Screening. *New Biotechnology. Elsevier* , Vol. 33 / S, 81-88
[16] A. Venkat Narsaiah, A. R. Reddy, B. V.S. Reddy, J. S. Yadav , (2011), Amberlyst-15: An Efficient, Cost-Effective and Recyclable Hetero Geneous Solid Acid Catalyst for the Synthesis of β-Enaminones and β- Enamino Esters, *The Open Catalysis Journal*, 4, 43-46.
[17] S.S.Pandit, S.K. Bhalerao, U.S.Aher, G.L.Adhav, V.U.Pandit, (2011), Amberlyst A-15: Reusable catalyst for the synthesis of 2,4,5-trisubstituted

and 1,2,4,5-tetrasubstituted-1*H*-imidazoles under MW irradiation., *j chem. Sci.*, 123(4), 421-426

[18] Performance Standards for Antimicrobial Disk Susceptibility Tests, 13th Edition, CLSI, M02, Wayne, PA, USA 2018

[19] Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 11th Edition, CLSI, M07, Wayne, PA, USA 2018