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Synthesis, Characterization and *In-vitro* evaluation of Antibacterial Profile of 1, 4-dihydropyridine derivatives

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A B S T R A C T

The objective of the present work was the synthesis of 1-[5-acetyl-4 (4-substituted phenyl)-2, 6-dimethyl-1,4-dihydroxypyridine-3-yl]-ethan-1-one and evaluation of *in vitro* antibacterial activity. Based on this a new series of compound had been planned to synthesize by reacting acetyl acetone with various aromatic aldehydes in the presence of ammonium acetate. The *in-vitro* antibacterial activity was carried out by Paper disc diffusion method and MIC was determined by Agar streak dilution method. The results displayed that all the synthesized compounds had a potential antibacterial activity against different gram positive and gram negative bacteria with an MIC range of 9-22 µg/ml (gram positive organisms) and with an MIC range of 10-26 µg/ml (gram negative organisms). The MIC of the synthesized compounds (B1, B2, B3, B4, B5 and B6) for different gram positive bacteria were found to be S. aureus (MIC: 9-18 µg /ml), B.subtilis (MIC: 10-21 µg /ml), S. typhi (MIC: 11-22 µg /ml) and S. epidermidis (MIC:12-21 µg /ml). The synthesized compounds (B1, B2, B3, B4, B5 and B6) were active against all the tested gram negative microorganisms with the range of MIC values for P. aeruginosa (MIC: 10-20 µg/ml), P. flurocense (MIC:12-25 µg/ml) and E. coli (MIC: 11-26 µg/ml) and V. cholerae (MIC:12-23 µg/ml).

Keywords: IR, NMR, Antibacterial activity, Paper disc diffusion, MIC

A R T I C L E I N F O

CONTENTS

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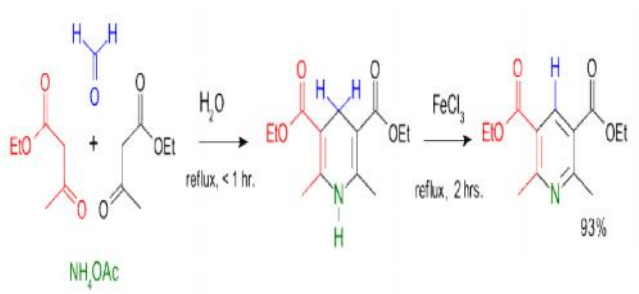
1. Introduction

Dihydropyridine is a molecule based upon pyridine, and the parent of a class of molecules that have been semi-saturated with two substituents replacing one double bond. They are particularly well known in pharmacology as L-type calcium channel blockers, used in the treatment of hypertension. Compared with certain other L-type calcium channel blockers (for example those of the phenylalkylamine class such as Verapamil) which have significant action at the heart, they are relatively vascular selective in their mechanism of action in lowering blood pressure [1].

The Hantzsch pyridine synthesis or Hantzsch dihydro pyridine synthesis is a multi-component organic reaction between an aldehyde such as formaldehyde, 2 equivalents of a β-keto ester such as ethyl acetoacetate and a nitrogen donor such as ammonium acetate or ammonia **[2].** The initial reaction product is a dihydropyridine which can be oxidized in a subsequent step to a pyridine. The driving force for this second reaction step is aromatization. This reaction was reported in 1881 by Arthur Rudolf Hantzsch. A 1, 4-dihydropyridine dicarboxylate is also called a 1, 4-DHP compound or a Hantzsch compound. These compounds are an important class of calcium channel blockers and as such commercialized in for instance nifedipine, amlodipine or nimodipine. The reaction has been demonstrated to proceed in water as reaction solvent and with direct aromatization by ferric chloride, Manganese Dioxide or potassium permanganate in a one-pot synthesis[3].



The Hantzsch dihydro pyridine synthesis is found to benefit from microwave chemistry [4]. Calcium channel blockers (CCB), calcium channel antagonists or calcium antagonists **[5]** are several medications that disrupt the movement of calcium (Ca2+) through calcium channels **[6]** Calcium channel blockers are used as antihypertensive drugs, i.e., as medications to decrease blood pressure in patients with hypertension. CCBs are particularly effective against large vessel stiffness, one of the common causes of elevated systolic blood pressure in elderly patients **[7]** Calcium channel blockers are also frequently used to alter heart rate, to prevent cerebral vasospasm, and to reduce chest pain caused by angina pectoris. N-type, L-type, and T-type voltage-dependent calcium channels are present in the zona glomerulosa of the human adrenal, and CCBs can directly influence the biosynthesis of aldosterone in adrenocortical cells, with consequent impact on the clinical treatment of hypertension with these agents [8].

Dihydropyridine (DHP) calcium channel blockers are derived from the molecule dihydropyridine and often used to reduce hypotension can lead systemic vascular resistance and arterial pressure. Sometimes when they are used to treat angina, the vasodilation and to reflex tachycardia, which can be detrimental for patients with ischemic symptoms because of the resulting increase in myocardial oxygen demand. Dihydropyridine calcium channel blockers can worsen proteinuria in patients with nephropathy [9]. Moreover 4-phenyl substituted 3,5–diacetyl–1,4-dihydro-pyridines Showed cytotoxic activity against human oral suamous carcinoma (HSC-2) cells [10, 11].

The objective of the present work is the synthesis of 1-[5-acetyl-4 (4-substituted phenyl)-2,6-dimethyl-1,4-dihydroxy pyridine-3-yl]-ethan-1-one and evaluation of *in vitro* antibacterial activity. Based on this a new series of compound have been planned to synthesize by reacting acetyl acetone with various aromatic aldehydes in the presence of ammonium acetate which involved Knoevena gel Condensation and Michael Addition reaction.

2. Materials and Methods

Chemicals and drugs

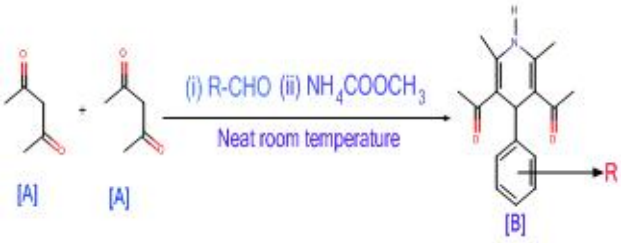
The all chemicals used for the synthesis were of laboratory grade and analytical grade. The melting points of newly synthesized 1,4-dihydrpyridine compounds were determined by open capillary method. The IR spectra of synthesized compounds were recorded by ABB Bomen FT-IR spectrometer MB 104 IR spectra recorder with KBr pellets**.** The H^1-NMR spectra of synthesized compounds were recorded by BRUKER NMR spectrometer in $CDCl_3$. The Mass spectra of synthesized compounds were recorded by JEOL GCmate. The purification of newly synthesiszed compounds were done by TLC method.TLC plates are pre-coated silica gel(HF254-200 mesh) aluminium plate using ethyl acetate and n-hexane as an solvent system and spots were visualized under U.V chamber. The IR, H^1-NMR and Mass spectra were assigned to elucidate the structure of synthesized compounds **(B1-B6).** Standard drugs Tetracyclin (TCLN) and Gentamicin (GENTA) were purchased from Local Retail Pharmacy Shop and solvents and other chemicals were used from Institutional Store and were of AR grade. Bacterial cultures Staphylococcus aureus (ATCC 9144), Bacilus subtilis (ATCC 6633), Salmonella typhi (ATCC 700931), Streptococcus epidermidis (ATCC 12228), Pseudomonas aeruginosa (ATCC 27853, Pseudomonas flurocense (ATCC13525),) Escherichia coli (ATCC 25922) and Vibrio cholerae (ATCC 14035) were provided by the Biotechnology Lab of

the CLBMCP, Chennai and maintained on Nutrient agar slant was maintained at 4^0 C.

General procedure for the synthesis of target compounds [12, 13]

To a stirred mixture of aromatic aldehydes (0.318 gm) and acetyl acetone (0.696 gm), ammonium acetate (0.231 gm) was added; the reaction mixture was homogenized by stirring to a viscous liquid. The progress of the reaction was monitored by TLC. The mobile phase for the synthesized compounds **B1, B2, B3, B4, B5 and B6** was ethyl acetate and n-hexane in the ratio of **6:4**. After completion of reaction a small amount of ethanol was added to the viscous liquid and stirred for 5 min. Ice cold water was added to the mixture, the solid thus obtained was filtered. The crude product was purified by crystallization from **ethanol: water (95:5)** mixture.

Synthetic scheme



[A] = Acetyl acetone.

[B] = Product (1, 4-dihydropyridine derivatives) - B1, B2, B3, B4, B5 and B6 etc.

Characterization of the synthesized compounds

Compound B1:

1 - [5-acetyl-4 (4-chloro phenyl)-2, 6-dimethyl-1, 4-dihydroxypyridine-3-yl] ethan-1-one.

Molecular formula: $C_{17}H_{18}ClNO_2$, Molecular weight: 303.102, m .p: 180-184 ^{0}C, yield: 85.2%. IR (KBr) cm^{-1}: 3334 (-NH str), 3078 (Ar-C-H), 2924 (C-H Aliph-C-H str), 1695 (C=O), 1582 (C=C), C-C (773), 1089 (C-N), 789 (C-Cl). ^{1}H-NMR ($CDCl_3$): δ 2.02 (s, 3H, CH_3), 2.9 (s, 3H, CH_3), 2.24 (s, 3H, $COCH_3$), 2.44 (s, 3H, $COCH_3$), 4.80 (s, 1H, CH), 7.18-8.82 (m, 7.18- 8.82-m, 4H, Ar-CH), 10.14 (s, 1H, NH). MS: m/z value-303.783. $(M)^+$ Ion peak.

Compound B2:

1 - [5-acetyl-4 (4-fluro phenyl)-2, 6-dimethyl-1, 4-dihydroxypyridine-3-yl] ethan-1-one.

Molecular formula: $C_{17}H_{18}FNO_2$, Molecular weight: 287.132, m .p: 175-179 ^{0}C, yield: 73.4%. IR (KBr) cm^{-1}: 3333 (-NH str), 3079 (Ar-C-H), 2923 (C-H Aliph-C-H str), 1695 (C=O), 1580 (C=C), C-C (775), 1088 (C-N), 688 (C-Cl). ^{1}H-NMR ($CDCl_3$): δ 2.01 (s, 3H, CH_3), 2.7 (s, 3H, CH_3), 2.23 (s, 3H, $COCH_3$), 2.42 (s, 3H, $COCH_3$), 4.81 (s, 1H, CH), 7.19-8.83 (m, 7.19- 8.84-m, 4H, Ar-CH), 10.13 (s, 1H, NH). MS: m/z value-287.32. (M)+ Ion peak.

Compound B3:

1 - [5-acetyl-4 (4-bromo phenyl)-2, 6-dimethyl-1, 4-dihydroxypyridine-3-yl] ethan-1-one.

Molecular formula: $C_{17}H_{18}BrNO_2$, Molecular weight: 347.052, m.p: 179-183 ^{0}C, yield: 69.9%. IR (KBr) cm^{-1}: 3334 (-NH str), 3070 (Ar-C-H), 2926 (C-H Aliph-C-H str), 1698 (C=O), 1581 (C=C), C-C (773), 1087 (C-N), 682 (C-Cl). ^{1}H-NMR ($CDCl_3$): δ 2.03 (s, 3H, CH_3), 2.9 (s, 3H, CH_3), 2.22 (s, 3H, $COCH_3$), 2.44 (s, 3H, $COCH_3$), 4.80 (s, 1H, CH), 7.19-8.84 (m, 7.19- 8.84-m, 4H, Ar-CH), 10.11 (s, 1H, NH). MS: m/z value-348.234 (M+1) Ion peak.

Compound B4:

1 - [5-acetyl-4 (4-nitro phenyl)-2, 6-dimethyl-1, 4-dihydroxypyridine-3-yl] ethan-1-one.

Molecular formula: $C_{17}H_{18}N_2O_4$, Molecular weight: 314.12, m.p: 145-147 ^{0}C, yield: 78.2%. IR (KBr) cm^{-1}: 3333 (-NH str), 3078 (Ar-C-H), 2925 (C-H Aliph-C-H str), 1696 (C=O), 1582 (C=C), C-C (771), 1088 (C-N), 1342 (NO_2 grp). ^{1}H-NMR ($CDCl_3$): δ 2.01 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.24 (s, 3H, $COCH_3$), 2.44 (s, 3H, $COCH_3$), 4.81 (s, 1H, CH), 7.19-8.83 (m, 7.19- 8.83-m, 4H, Ar-CH), 10.15 (s, 1H, NH). MS: m/z value-314.33 (M)+ Ion peak.

Compound B5:

1- [5-acetyl-4 (4-Hydroxy phenyl)-2, 6-dimethyl-1,4-dihydroxypyridine-3-yl] ethan-1-one. Molecular formula: $C_{17}H_{19}NO_3$, Molecular weight: 285.136, m .p: 90-94^{0}C, yield: 81.3%. IR (KBr) cm^{-1}: 3334 (-NH str), 3078 (Ar-C-H), 2924 (C-H Aliph-C-H str), 1695 (C=O), 1582 (C=C), C-C (773), 1089 (C-N), 789 (C-Cl). ^{1}H-NMR ($CDCl_3$): δ 2.19 (s, 3H, CH_3), 2.9 (s, 3H, CH_3), 2.22 (s, 3H, $COCH_3$), 2.43 (s, 3H, $COCH_3$), 3.97 (s,4H,), 7.01-7.43 (4H, Ar-CH), 10.14 (s, 1H, NH). MS: m/z value-285.3 $(M)^+$ Ion peak.

Compound B6:

1-[5-acetyl-4 (4-methoxy phenyl)-2, 6-dimethyl-1, 4-dihydroxypyridine-3-yl] ethan-1-one.

Molecular formula: $C_{18}H_{21}NO_3$, Molecular weight: 299.15, m .p: 102-105^{0}C, yield: 77.2%. IR (KBr) cm^{-1}: 3433 (-NH str), 2992 (Ar-C-H), 2961 (C-H Aliph-C-H str), 1629 (C=O, $COCH_3$), 1574 (C=C), C-C (794), 1034 (C-N). ^{1}H-NMR ($CDCl_3$): δ 1.93 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 2.73 (s, 3H, $COCH_3$), 2.78 (s, 3H, $COCH_3$), 3.97 (s, 3H, OCH_3), 4.62 (s, 1H, CH) 7.04-7.43 (m, 3H, Ar-CH), 10.14 (s, 1H, NH). MS: m/z value-299.36 $(M)^+$ Ion peak.

Evaluation of invitro antibacterial profile by Paper disc diffusion method [14]

The sterilized (autoclaved at 120 ^{0} C for 30 min) medium was inoculated (1 mL/100mL of medium) with the suspension [105 cfu m/l (colony forming unit per milliliter)] of the microorganism (matched to McFarland barium sulphate standard) and poured in Petri dish to give a depth of 3-4mm. The paper impregnated with the test compounds (25, 50, 100,150 µg/ml in dimethyl formamide) was placed on the solidified medium. The plates were pre-incubated for 1hr at RT and incubated at 37^o C for 24 hr for anti-bacterial activity respectively. Tetracyclin and Gentamicin (100 µg/disc) were used as a standard drugs for gram-positive and gram-negative bacteria respectively. The observed zone of inhibition was compared with standard drugs.

Determination of MIC by Agar streak dilution method [15]

MIC of the synthesized compounds was determined by agar streak dilution method. A stock solution of the compound (100µg/ml) in Dimethyl formamide was prepared and graded quantities of the test compound was incorporated in specified quantities of molten nutrient agar medium. A specified quantity of the medium containing the test compound was poured into a Petri dish to give a depth of 3-

4mm and allowed to solidify. Suspension of the micro-organism were prepared to contain approximately105 cfu m/l and applied to plates with serially diluted extracts in Dimethyl formamide to be tested and incubated at 37° C for 24hr. for bacteria. The MIC was considered to be the lowest concentration of the test substance exhibiting no visible growth.

3. Results and Discussion

The synthesized compounds B1, B2, B3, B4, B5 and B6 were (25, 50, 100 and 150µg/ml) were screened for antibacterial activity by paper disc diffusion method. From the data shown in Table 1 and 2 the observations were made as followed: Most of the synthesized compounds executed moderate to good antibacterial activity against the tested micro-organisms. When compared to standard drug Tetracyclin (TCLN), the synthesized compounds B1, B2, B3, B4, B5 and B6 were found to exhibit good Anti-bacterial activity against various gram positive bacteria. When compared to standard drugs Gentamicin (GENTA), the synthesized compounds B1, B2, B3, B4, B5 and B6 were found to exhibit good Antibacterial activity against various gram negative bacteria.

The MIC of the synthesized compounds (B1, B2, B3, B4, B5 and B6) was screened by agar streak dilution method. From the data shown in Table 3 and 4 observations were made as followed: All the synthesized compounds (B1, B2, B3, B4, B5 and B6) exhibited moderate to good antibacterial activity with an MIC range of 9-22 µg/ml (gram positive organisms) and with an MIC range of 10-26 µg/ml (gram negative organisms). The MIC values of the synthesized compounds (B1, B2, B3, B4, B5 and B6) for different gram positive bacteria were found to be S. aureus (MIC: 9-18 µg /ml), B.subtilis (MIC: 10-21 µg /ml), S. typhi (MIC: 11-22 µg /ml) and S. epidermidis (MIC:12-21 µg /ml). The synthesized compounds (B1, B2, B3, B4, B5 and B6) were active against all the tested gram negative microorganisms with the range of MIC values for P. aeruginosa (MIC: 10-20 µg/ml), P. flurocense (MIC: 12-25 µg/ml) and E. coli (MIC: 11-26 µg/ml) and V. cholerae (MIC:12-23 µg/ml).

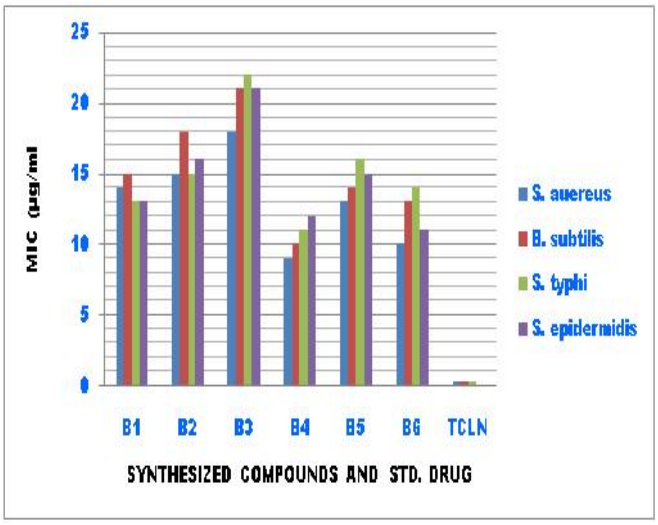


Figure 1: Graphical representation of MIC of synthesized compounds and std. drug Tetracyclin (TCLN) against different gram positive bacteria and gram negative bacteria S. typhi.

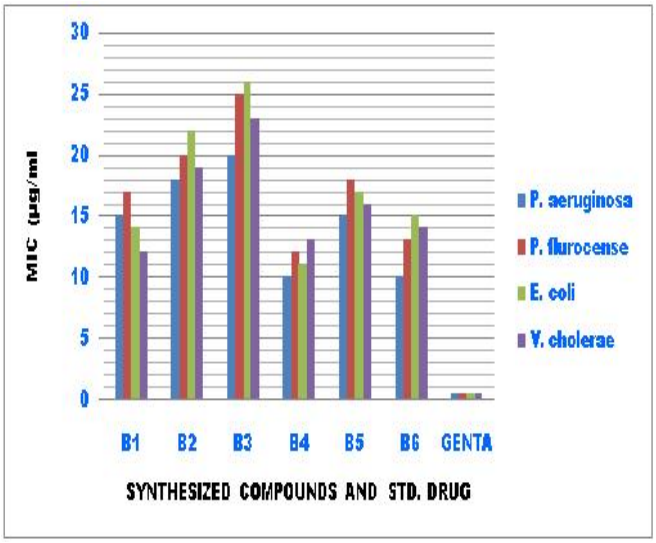


Figure 2: Graphical representation of MIC of synthesized compounds and std. drug GENTA (Gentamicin) against different gram negative bacteria.

Table 1: For Zone of Inhibition (mm) of Gram-positive Bacteria and gram negative bacteria S. typhi by Synthesized compounds (B1; B2; B3; B4; B5 & B6).

Name of the Compounds	S. aureus				B. subtilis				S. typhi				S. epidermidis			
	Concentration of B1; B2; B3; B4; B5 & B6(µg/ml)															
	25	50	100	150	25	50	100	150	25	50	100	150	25	50	100	150
	Zone of inhibition(mm) at different concentration															
B1	5	10	15	17	3	9	17	19	6	7	16	18	4	8	18	20
B2	4	9	16	16	5	9	18	20	5	8	17	20	4	9	19	21
B3	6	8	13	15	6	10	16	19	5	9	18	21	6	10	20	22
B4	4	12	20	24	5	11	19	22	4	10	19	22	5	9	19	23
B5	4	10	14	16	4	9	18	17	4	6	17	19	5	7	19	21
B6	5	8	13	14	3	11	16	19	6	7	15	20	5	8	17	19
TCLN	28 (100µg/ml)				28 (100µg/ml)				28 (100µg/ml)				28 (100µg/ml)			

Table 2: For Zone of Inhibition (mm) of Gram-negative Bacteria by Synthesized compounds (B1; B2; B3; B4; B5 & B6)

Name of the compounds	P. aeruginosa				P. flurocense				E.coli				V. cholerae			
	Concentration of B1; B2; B3; B4; B5 & B6 (µg/ml)															
	25	50	100	150	25	50	100	150	25	50	100	150	25	50	100	150
	Zone of inhibition(mm) at different concentration															
B1	5	9	14	19	3	9	16	19	6	7	16	18	4	8	18	20
B2	4	8	16	18	5	7	15	20	5	8	17	20	4	9	17	21
B3	6	7	12	16	6	11	14	17	5	9	15	19	6	10	16	22
B4	4	11	20	22	5	12	19	21	4	10	19	22	5	9	20	23
B5	5	8	13	17	3	8	15	18	7	8	15	17	3	4	16	19
B6	6	8	19	20	7	10	17	19	6	7	18	19	5	7	19	21
GENTA	28 (100µg/ml)				28 (100µg/ml)				28 (100µg/ml)				28 (100µg/ml)			

Table 3: For MIC of Synthesized compounds (B1; B2; B3; B4; B5 & B6) against different gram positive bacteria and gram negative bacteria S. typhi

Compounds	Minimum Inhibitory Concentration (MIC) [µg/ml]			
	S. auereus	B. subtilis	S. typhi	S. epidermidis
B1	14	15	17	13
B2	15	18	15	16
B3	18	21	22	21
B4	9	10	11	12
B5	13	14	16	15
B6	10	13	14	11
TCLN	0.2	0.2	0.2	0.2

Table 4: For MIC of Synthesized compounds (B1; B2; B3; B4; B5 & B6) against different gram negative bacteria

Compounds	Minimum Inhibitory Concentration (MIC) [µg/ml]			
	P. aeruginosa	P. flurocense	E. coli	V. cholerae
B1	15	17	14	12
B2	18	20	22	19
B3	20	25	26	23
B4	10	12	11	13
B5	15	18	17	16
B6	10	13	15	14
GENTA	0.5	0.5	0.5	0.5

4. Conclusion

From the present study it was concluded that most of the synthesized compounds executed moderate to good antibacterial activity against tested microorganisms (Gram positive and Gram negative bacteria) with an MIC range of 9-22 µg/ml (gram positive organisms) and with an MIC range of 10-26 µg/ml (gram negative organisms). The MIC of the synthesized compounds (B1, B2, B3, B4, B5 and B6) for different gram positive bacteria were found to be S. aureus (MIC: 9-18 µg /ml), B.subtilis (MIC: 10-21 µg /ml), S. typhi (MIC: 11-22 µg /ml) and S. epidermidis (MIC:12-21 µg /ml). The synthesized compounds (B1, B2, B3, B4, B5 and B6) were active against all the tested gram negative microorganisms with the range of MIC values for P. aeruginosa (MIC: 10-20 µg/ml), P. flurocense (MIC:12-25 µg/ml) and E. coli (MIC: 11-26 µg/ml) and V. cholerae (MIC:12-23 µg/ml).

5. References

1. 1, 4-dihydropyridine - Compound Summary". *Pubchem Compound*. USA: National Center for Biotechnology Information. 27 March 2005. Identification and Related Records. Retrieved 1 November 2011.
2. Hantzsch, A. Condensation produkteaus Aldehyd ammonia and Ketonartigen Verbindungen. Chemische Berichte. **1881,** 14(2): 1637–8.
3. Xia, J. J.; Wang, G. W. (2005). "One-Pot Synthesis and Aromatization of 1, 4-Dihydropyridines in Refluxing Water". Synthesis. **2005,** 14: 2379–83.
4. Van den Eynde, J. J.; Mayence, A. "Synthesis and Aromatization of Hantzsch 1,4-Dihydropyridines

under Microwave Irradiation. An Overview". Molecules. **2003**, **8** (4): 381–91.

5. Olson, Kent (2011). "40. Calcium Channel Antagonists". *Poisoning & drug overdose* (6th ed.). McGraw-Hill Medical. ISBN 0071668330.
6. Nelson M (2010). "Drug treatment of elevated blood pressure" (pdf). *Australian Prescriber* **33** (4): 108–112
7. Felizola SJA, Maekawa T, Nakamura Y, Satoh F, Ono Y, Kikuchi K, Aritomi S, Ikeda K, Yoshimura M, Tojo K, Sasano H. Voltage-gated calcium channels in the human adrenal and primary aldosteronism. *J. Steroid Biochem Mol Biol.* **2014**, **144** (part B): 410–6.
8. Remuzzi G, Scheppati A, Ruggenenti P (2002). "Clinical Practice. Nephropathy in Patients with Type 2 Diabetes". *New England Journal of Medicine*. 2002, **346** (15): 1145–51.
9. Godfraid. T, Miller. R, Wibo. M, Calcium antagonist and calcium entry blockade of 1, 4-dihydropyridine, Pharmacology Review, **1986**, 38: 321-416.
10. Rudong Shan, Howelett. E, E. Knaus, Synthesis, Calcium channel agonist-antagonist modulation activities, nitric oxide release and voltage-clamp studies of 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl -3-nitro-4-(2-trifluro methyl phenyl) pyridine-5-carboxylate enantiomers. Journal of Medicinal Chemistry, **2002**, 45(4): 955-961.
11. Maitland Jones. Jr, Text book of Organic chemistry. 2nd Edition. New York, London. W.W Norton & Company. **2000**. Pp. 1241-1242.
12. Norman R.O.C, Coxon J. M, Principal of organic synthesis. 3rd Edition. 1st Indian reprint **2009**. C.R.C Press. P. 699-702.
13. R. S. Gaud, G. D. Gupta "Practical microbiology" 3rd edition. **2004**: 41.
14. Hawkey BY, Lewis DA, Medical microbiology- a practical approach. United Kingdom: Oxford, university, press; **1994**, pp.181-94.