Synthesis, Characterization and Biological Evaluation of Few Hydroxamic Acid Derivatives

Thesis submitted by

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Kolkata, India

2019

JADAVPUR UNIVERSITY

KOLKATA-700 032, INDIA

INDEX NO. 133/12/Ph

1. Title of the thesis:

Synthesis, Characterization and Biological Evaluation of Few Hydroxamic Acid Derivatives.

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3. List of Publication:

A. Related to the thesis work (2)

1. **Das M**, Das B, De A, Banerjee S, Samanta A. Antimicrobial investigation and binding mode analysis of some newly Synthesized 4-amino-5-((aryl substituted)-4h-1, 2, 4-triazole-3-yl)-thio linked Hydroxamic acid derivatives, Asian J Pharm Clin Res, 2019, 12(3): 469-479.
2. **Das M**, Das B, Samanta A. Antioxidant and anticancer activity of synthesized 4-amino-5-((aryl substituted)-4H-1,2,4-triazole-3-yl) thio-linked hydroxamic acid derivatives. Journal of Pharmacy and Pharmacology, 2019; 71: 1400–1411. (doi: 10.1111/jphp.13131).

B. Other publications during the period of doctoral research (15)

1. Mandal S, **Das M**, Das P, Samanta A, Butcher RJ, Saha M, Alswaidan IA, Rhyman L, Ramasami P, Saha NC. Synthesis, characterization, DFT and antimicrobial studies of transition metal ion complexes of a new schiff base ligand, 5-methylpyrazole-3yl-N-(2́-hydroxyphenylamine) methyleneimine,(MPzOAP). Journal of Molecular Structure. 2019; 1178:100-11.
2. Das B, De A, **Das M**, Das S, Samanta A. A new exploration of Dregea volubilis flowers: Focusing on antioxidant and antidiabetic properties. South African Journal of Botany, 2017; 109: 16–24.

3. Bandyopadhyay N, **Das M**, Samanta A, Zhu M, Lu L, Naskar JP. Promising Antimicrobial Activity of an Oxime Based Palladium(II) Complex. Chemistry Select, 2017; 2; 230-40.

4. Mitra D, Basu A, Das B, Jena AK, De A, **Das M**, Bhattacharya S, Samanta A. Gum odina: an emerging gut modulating approach in colorectal cancer prevention. RSC Advances. 2017; 7(46): 29129-42.

5. Kundu S, Abdullah MF, Das A, Basu A, Halder A, **Das M**, Samanta A, Mukherjee A. Antifungal ouzo nanoparticles from guar gum propionate. RSC Advances. 2016; 6(108):106563-71.

6. Mitra D, Jena AK, De A, **Das M**, Das B, Samanta A. Prebiotic potential of gum odina and its impact on gut ecology: in vitro and in vivo assessments. Food & function. 2016; 7(7):3064-72.

7. Bandyopadhyay N, Zhu M, Lu L, Mitra D, **Das M**, Das P, Samanta A, Naskar JP. Synthesis, structure, spectral characterization, electrochemistry and evaluation of antibacterial potentiality of a novel oxime-based palladium (II) compound. European journal of medicinal chemistry. 2015; 89:59-66.

8. Mandal A, Ojha D, Lalee A, Kaity S, **Das M**, Chattopadhyay D, Samanta A. Bioassay directed isolation of a novel anti-inflammatory cerebroside from the leaves of Aerva sanguinolenta. Medicinal Chemistry Research. 2015; 24(5):1952-63.

9. Jena AK, **Das M**, De A, Mitra D, Samanta A. Determination of Efficacy of a Natural Tablet Binder: Characterization and in- vitro Release Study. Asian Journal of Pharmaceutical and Clinical Research, 2014; 7(3): 164-8.

10. Saha NC, Mandal S, **Das M**, Khatun N, Mitra D, Samanta A, Slawin AM, Butcher RJ, Saha R. Synthesis, characterization, X-ray crystallography and antimicrobial activities of new Co (III) and Cu (II) complexes with a pyrazole based Schiff base ligand. Polyhedron. 2014; 68:122-30.

11. Saha NC, Pradhan R, **Das M**, Khatun N, Mitra D, Samanta A, Slawin AM, Jana AD, Klanke J, Rentschler E. Synthesis, characterization, X-ray crystallography, and antimicrobial activities of Ni (II) and Cu (II) complexes with a salicylaldehyde-based thiosemicarbazone ligand. Journal of Coordination Chemistry. 2014; 67(2):286-99.

12. Biswas C, Zhu M, Lu L, Kaity S, **Das M**, Samanta A, Naskar JP. A palladium (II) complex: Synthesis, structure, characterization, electrochemical behavior, thermal aspects, BVS calculation and antimicrobial activity. Polyhedron. 2013; 56:211-20.

13. Sarkar M, **Das M**, Mitra D, Jena AK, De A, Samanta A. Anticancer potential of methanolic and aqueous extract of Leucasindica (Linn.) against ehrlich ascites carcinoma cells on swiss albino mice International research journal of pharmacy 2013; 4(6):183-8.

14. Lalee A, Bhattacharaya B, **Das M**, Mitra D, Kaity S, Bera S, Samanta A. Hepatoprotective activity of ethanolic extract of Aerva sanguinolenta (Amaranthaceae) against paracetamol induced liver toxicity on Wistar Rats. NSHM Journal of Pharmacy and Healthcare Management. 2012; 3:57-65.

15. Samanta A, Pal P, Mandal A, Sinha C, Lalee A, **Das M**, Kaity S, Mitra D. Estimation of biosurfactant activity of an alkaline protease producing bacteria isolated from municipal solid waste. Cent. Eur. J. Exp. Biol. 2012; 1:26-35.

4. List of Patents: Nil.

5. List of Presentations in National/International/ Conferences/ Workshop: (V)

A. National

I. Seminar on "Recent advances in Clinical Research with a special emphasis on BA/ BE study" at Jadavpur University, Kolkata, West Bengal, India on 2014.

II. National workshop on "Revisiting Intellectual Property Rights in the Context of Recent Developments in Science & Technology" at Jadavpur University , Kolkata, West Bengal, India on October 20, 2016.

III. GCP Workshop conducted by Department of Hematology, Nil Ratan Sircar Medical College and Hospital and Novartis Oncology on 21st April 2017 at Kolkata, India.

B. International

I. 2nd Pharm. Tech. IAPST International Conference on "New Insights into Diseases and Recent Therapeutic Approaches", Jadavpur University, W.B., India on January 2014.

II. Poster presentation at "The 1st International Conference of pharmacy education and research network of ASEAN" organized by Mahidol University, Bangkok, Thailand on December 2015.

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Synthesis, Characterization and Biological Evaluation of Few Hydroxamic Acid Derivatives**" submitted by Smt **Mousumi Das,** who got her name registered on **02/05/2012** for the award of **Ph. D. (Pharmacy)** degree of Jadavpur University is absolutely based upon her own work under the supervision of **Prof.** (**Dr.) Amalesh Samanta**, Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata-700032 and that neither her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

(Signature of Supervisor)
Prof. (Dr.) Amalesh Samanta
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DECLARATION

This research work entitled "**Synthesis, Characterization and Biological Evaluation of Few Hydroxamic Acid Derivatives**" has been carried out by me under the supervision of **Prof. (Dr.) Amalesh Samanta** in the Department of Pharmaceutical Technology, Faculty Council of Engineering and Technology, Jadavpur University, Kolkata-700 032, India. I hereby declare that the work is original and has not been submitted so far, in part or full for any degree/diploma courses of any University.

Date: Full signature of the candidate

Place: (MOUSUMI DAS)

ACKNOWLEDGEMENTS

Coming to this stage of my academic carrier, I would like to acknowledge each and everyone, not only those who have supported me during this years of my life but also those who have always kept believe in me.

First of all, I would like to thank my Professor, **Prof. (Dr.) Amalesh Samanta** for giving me an opportunity to work under his guidance. He always had the confidence in me and my abilities, which encourage me to grow as a future researcher. I convey my sincere regards and deepest gratitude to him.

I remain deeply indebted to the Dean of the Faculty of Engineering and Technology and Head of the Department of Pharm. Tech. of Jadavpur University for providing me with all the necessary facilities to carry out this work.

I want to thank **Dr. Subhasis Banerjee**, who was my supervisor during the M. Pharm. degree course. He always helped me in my tough times.

I would also like to thank my lab mates, **Dr. Debmalya Mitra, Piu Das, Bhaskar Das, Arnab De**, my seniors, **Dr. Soma Ghosh, Dr. Mahananda Sarkar, Dr. Sayantan Moitra, Dr. Anurup Mandal, Dr. Aditya Kr. Jena, Dr. Partha Sarathi Roy, Dr. Samit Bera,** and my other lab colleagues **Shilpa Das, Paloma Patra, Ahana Hazra, Mousumi Tudu, Partha Pratim Biswas** for their friendship, motivation and sharing of knowledge and experiences.

I dedicate this thesis to my parents, sister and my dear husband for their unconditional love, patience and support. They are always present in all the important moments of my life for making me the person that I am today.

Dedicated to my beloved parents, my sweet sister

and my dear husband and together I

call it, family

CONTENTS

Preface

Triazole is getting more important nowadays due to its high efficacy and better safety profiles. Several synthetic routes have been discovered for the synthesis of triazole and they are economically advantageous with their low production cost. Due to their wide range of biological, agrochemical and chemical properties, they have drawn the attention to the scientists. Triazoles have a broader spectrum of applications compared to imidazoles leading to this class of azoles being. 1,2,4-Triazole derivatives have different biological activities, like as antifungals (Itraconazole, Fluconazole), anticancer, in migraine headaches (Rizatriptan), anti-inflammatory, antihypertensive etc. In the area of coordination chemistry, hydroxamic acids are excellent ligands. They deprotonate to give hydroxamates, which bind to metals ions as bidentate ligands. So high is the affinity of hydroxamates for ferric ions that nature has evolved families of hydroxamic acids to function as iron-binding compounds (siderophores) in bacteria. Hydroxamic acids are successful molecules in the field of cancer chemotherapy. Suberoylanilide Hydroxamic Acid (SAHA), Belinostat & panobinostat are the hydroxamates based drugs for cancer chemotherapy, approved by the U.S. Food and Drug Administration (FDA). Some others are also in clinical trials, like m-carboxycinnamic acid bishydroxamic acid, SB-939 (phase II) and 4SC-201. SAHA (Suberoylanilide Hydroxamic Acid) is considered as a potent anticancer agent among all hydroxamic acid derivatives because of their very good chelating capability, That's why the hydroxamates group grabs the attraction for scientists from all over the world.

Depending on the fact the subject matter of the thesis has been divided into several chapters covering the synthesis, chemical characterization and biological evaluation like, antibacterial, antifungal, antioxidant, anticancer and antileishmanial effect of some 1,2,4-triazole linked hydroxamic acid derivatives.

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ABBREVIATIONS

Å	: Angstrom
AIDS	: Acquired immune deficiency syndrome
ATCC	: American Type Culture Collection
b.w	: Body weight
CFU	: Colony forming unit
˚C	: Degree Celsius
CRE	: Creatinine
CL	: Cutaneous leishmaniasis
DMSO	: Dimethyl sulfoxide
DCM	: Dichloromethane
FTIR	: Fourier transform infrared spectroscopy
FCS	: Fetal Calf Serum
g	: Gram
h	: Hour
Hb	: Hemoglobin
i.e.	: That is
IC_{50}	: 50% inhibitory concentration
i.p.	: Intraperitoneal
LD_{50}	: Lethal dose 50
mg	: Milligram
min	: Minute

MIP	: Macrophage Inflammatory Protein
Ml	: Milliliter
Mw	: Molecular weight
MeOH	: Methyl alcohol
MIC	: Minimum inhibitory concentration
MS	: Mass spectrometry
MTT	: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NaOH	: Sodium hydroxide
NCTC	: National Collection of Type Culture
NMR	: Nuclear magnetic resonance
NSAIDS	: Non steroidal anti inflammatory drugs
OECD	: Organisation for Economic Co-operation and Development
PMM	: Paromomycin
ppm	: Parts-per million
PBS	: Phosphate buffer saline
P-value	: Probability value (in statistical significance testing)
RPMI	: Roswell Park Memorial Institute medium
SAHA	: Suberoylanilide hydroxamic acid
SEM	: Scanning electron microscopy
SGOT	: Serum glutamic - oxaloacetic transaminase
SGPT	: Serum glutamic - pyruvic transaminase
SD	: Standard deviation

SI	: Selectivity Index
SSG	: Sodium stibogluconate
TLC	: Thin layer chromatography
VL	: Visceral leishmaniasis
WHO	: World Health Organization
ZOI	: Zone of inhibition
µg	: Microgram
µl	: Microliter
µM	: Micromolar
mM	: Millimolar

Chapter 1

1. Introduction

1.1. Triazole

Triazole is getting more important nowadays due to its high efficacy across a variety of species and better safety profiles. Several synthetic routes have been discovered for the synthesis of triazole and they are economically advantageous with their low production cost. Triazole is a five-membered heterocyclic ring containing three nitrogen atoms at 1,2 and 4 positions. Triazole was first synthesized by Fischer in 1878. According to literature review, around 0.2 million 1,2,4-triazole derivatives have been reported. Due to their wide range of biological, agrochemical and chemical properties, this class of organic compounds has drawn the attention to the scientists (Maddila et al 2013). The synthesis and biological activity of 1,2,4-triazoles have been reviewed time to time (Potts et al. 1961, Ferreira et al. 2013, Holm et al. 2011, Bele et al. 2011, Hussain et al. 2016) For the treatment of invasive and systemic fungal infections there are a limited numbers of pharmaceuticals are available. Over the last 10 years with the addition of new antifungal medications into the market, the sales of the antifungal have increased (Gross et al. 2015). The total use of antifungal medication in United States hospitals increased from 2004-2007 (Pakyz et al. 2011). In 2010, near about USD 9.4 billion transactions were made. From 2002 to 2010 the annual growth rate for antifungal was 2.9% (Gross et al. 2015). In the systemic antifungal market, azoles account has been increasing since 1999. From 1999 to 2003, the sale for systemic antifungal was increased an approximate 11.4% depending on can various factors. In the case of intensive care, organ transplantation and oncology patients the triazoles show the promising role and the use of these pharmaceuticals adds to the risk of potential exposure to invasive fungal species (Harris et al. 2004). Over the last 20 years, a number of new fungi species were discovered and consequently the number of antifungal pharmaceuticals also increased (Montagna

et al. 2013). Imidazoles don't have activity against *Aspergillus* species, and therefore, are predominately used to treat superficial and mucosal infections (Pasqualotto et al. 2010). Triazoles have a broader spectrum of applications compared to imidazoles leading to this class of azoles being. The first-generation triazoles like itraconazole and fluconazole were found to the United States market and established in the suppression of fungal infections. About a decade later, the second-generation triazole antifungals (voriconazole, posaconazole, ravuconazole, albaconazole, isavuconazole, efinaconazole) were developed.

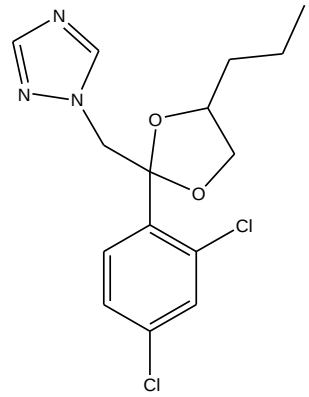
1.1.1. 1,2,3-Triazole

1,2,3-triazole is a basic aromatic heterocyclic (Gilchrist et al. 1997) with a molecular formula $C_2H_3N_3$. A reaction of azide and alkyne substituted 1,2,3-triazoles are produced (Huisgen cycloaddition). In comparison to other organic compounds which have three adjacent nitrogen atoms, it is a more stable structure. In aqueous solution, the tautomer form is 2H-1,2,3-triazole (Albert et al. 1989).

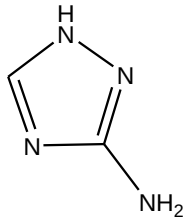
1.1.2. 1,2,4-Triazole

1,2,4-triazole is a basic aromatic heterocyclic with a molecular formula $C_2H_3N_3$. It is prepared by Einhorn–Brunner reaction or the Pellizzari reaction. 1,2,4-Triazole derivatives have different biological activities, especially as antifungals such as itraconazole, fluconazole. They were the first class of triazoles, but they had limited use. To overcome the problem derivatives of itraconazole or fluconazole like posaconazole, voriconazole, efinaconazole, albaconazole, ravuconazole and isavuconazole were discovered (Peyton et al. 2015). Moreover, 1,2,4-triazole derivatives are used as antiasthmatics (Naito et al. 1996), anticonvulsants (Küçükgüzel et al. 2004) antidepressants (Oruç et al. 1999), anti-inflammatory (Maddila et al. 2015) and insecticidal (Maddila et al. 2015). Moreover, compounds having triazole moieties, such as

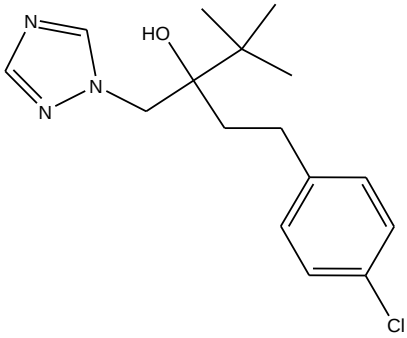
vorozole, letrozole and anastrozole are found to be the most active aromatase inhibitors, which prevent breast cancer (Santen et al. 2003, Clemons et al. 2004, Delea et al. 2008). Propiconazole is very potent broad-spectrum fungicides used in agriculture. 1,2,4-Triazole derivatives have been used as anticancer drugs. Rizatriptan, a derivative of 1,2,4 triazole is used for the treatment of migraine headaches are commercially available drugs. Three nitrogen donor atoms of 1,2,4-triazoles are present in their heterocyclic core which helps them to bind the terminal, bridging or chelating coordination modes. Thus, in the absence of substituents capable of coordination, 1,2,4-triazoles show a tendency towards bidentate bridging coordination, resulting in the formation of oligo- or polynuclear compounds. These polynuclear multi-dimensional arrays have been used as metal-organic frameworks (MOF) or as magnetic materials.



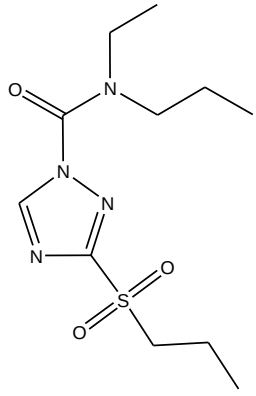
Propiconazole (Fungicide)



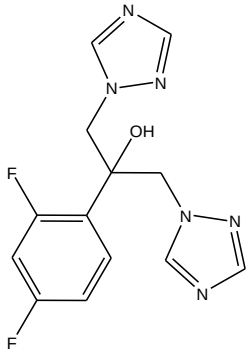
Amitrole (herbicide)



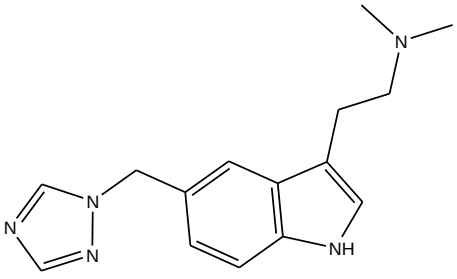
Tebuconazole (Fungicide)



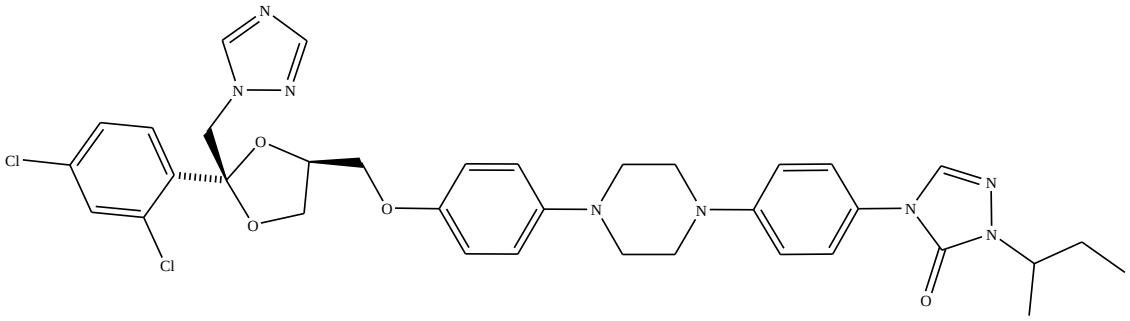
Epronaz (herbicide)



Fluconazole (antifungal drug)



Rizatriptan (treatment of migraine headaches)



Itraconazole (antifungal drug)

Fig 1. Examples of pesticides and drugs containing a 1,2,4-triazole fragment.

1.1.3. Synthesis of triazoles

Synthesis of triazoles was done based on their two isomers, 1,2,3- and 1,2,4-triazole.

1,2,3-Triazole Synthesis

1,2,3-triazole isomer is synthesized by the Huisgen Azide-Alkyne method. An azide is an organic compound having three nitrogen atoms bonded to a general R group, and an alkyne is an organic compound that has a carbon-carbon triple bond. When an azide and an alkyne is reacted together, a 1,2,3-triazole is formed.

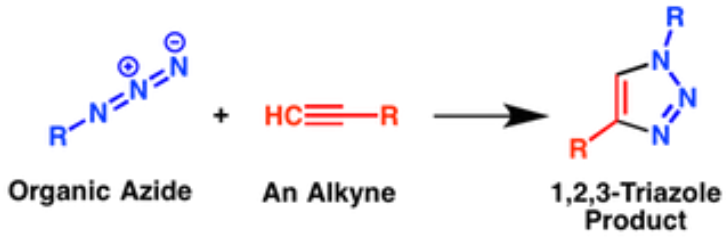


Fig 2. 1,2,3 -Triazole synthesis from an organic azide and an alkyne

1,2,4-Triazole Synthesis

1,2,4-triazole isomer is produced by reacting organic hydrazine with formamide and then microwave it. In a microwave sometimes organic reactions (a lot like popcorn) are run. Organic hydrazine contains a generic R group bonded to two nitrogen atoms, and formamide is a building

block that contains a carbonyl group (carbon-oxygen double bond) connected to a nitrogen atom and hydrogen.

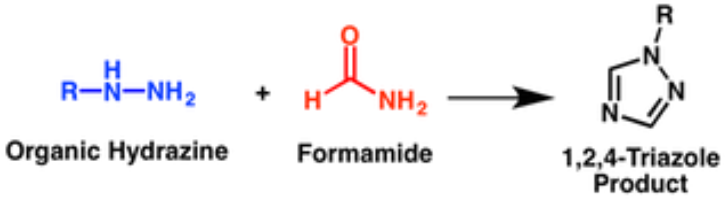


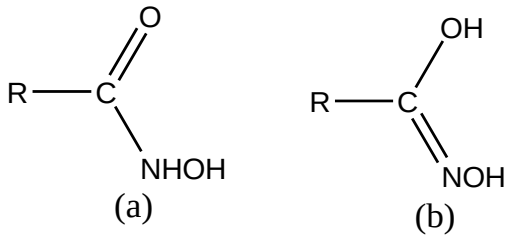
Fig 3. 1,2,4-Triazole synthesis from organic hydrazine and formamide

1.1.4. Mechanism of action and spectrum of activity

The triazoles have a similar mechanism of action and spectrum of activity as imidazoles. Fluconazole is used for the treatment of candidiasis and cryptococcal infection. In the case of mucocutaneous candidiasis, itraconazole is used. It is also used for dermatophyte infections, such as pityriasis versicolor (*Pittosporum orbicular or Malassezia furfur* organisms are responsible) and ringworms. Voriconazole is used for the treatment of invasive aspergillosis and *Scedosporium* species, *Fusarium* species causing serious infections, or fluconazole-resistant *Candida glabrata and* C. *krusei.*

1.2. Hydroxamic Acid

W. Lossen has first introduced the structure of the hydroxamic acids (Lossen et al. 1873) which include the interaction of anisohydroxamic acid and benzoyl chloride was different from the product obtained with benzohydroxamic acid and anisoyl chloride. Hydroxamic acids are N-monoacylhydroxylamines, RCONHOH, and exist in two forms, oxo- (a) and imino (b).



Hydroxamic acid is a class of organic compounds having the functional group RC(O)N(OH)R', where R and R' as organic residues and CO as a carbonyl group. They are amides (RC(O)NHR') wherein the NH centre has an OH substitution. They are frequently used as metal chelators. Some hydroxamic acids are HDAC inhibitors with anti-cancer properties like Trichostatin A, vorinostat, panobinostat and belinostat. In the area of coordination chemistry, hydroxamic acids are excellent ligands (Rahman et al., 2018). They deprotonate to give hydroxamates, which bind to metals ions as bidentate ligands. So high is the affinity of hydroxamates for ferric ions that nature has evolved families of hydroxamic acids to function as iron-binding compounds (siderophores) in bacteria. They dissolve insoluble iron(III) compounds. The resulting complexes are transported into the cell, where the iron is extracted and utilized metabolically (Chauhan et al., 2016). Ligands derived from hydroxamic acid and thiohydroxamic acid also form strong complexes with lead (II) (Farkas et al., 2017).

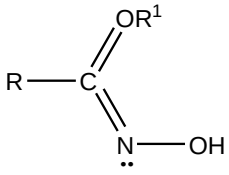
1.2.1. Present Status of the Chemistry of Hydroxamic Acids

Hydroxamic acids and their derivatives have a wide variety of biological activity. A number of hydroxamic acids have been found as analytical reagents. Reactions of hydroxamic acids and their derivatives have not been extensively studied and therefore, it is considered worthwhile to review briefly the present status of the knowledge in this field (Bauer et al. 1974).

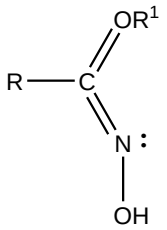
1.2.2. Structure of Hydroxamic Acid

Hydroxamic acids are solids and oily liquids, soluble in water up to a size of about six carbons and nearly insoluble in ether. These are weak acids comparable to phenol in strength. These are also weakly basic and form salts with strong acids in non-aqueous media. Hydroxamic acids are used extensively in the flotation of rare earth minerals during the concentration and extraction of

ores to be subjected to further processing. The benzo derivatives of hydroxamic acid are known as benzyl (z) – benzohydroximate.

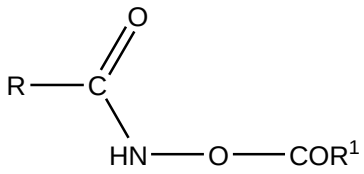


Benzyl

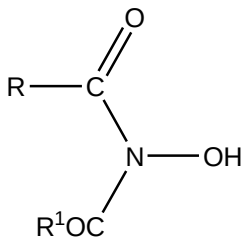


Benzohydroximate

The acyl hydroxamic acid derivatives are named as derivatives of O, N-diacylhydroxylamines and N - acetylacetohydroxamic acid respectively.



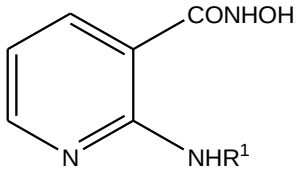
O, N-diacylhydroxylamines



N - acetylacetohydroxamic acid

By the reaction of substituted hydroxylamine and an acid chloride in pyridine corresponding substituted hydroxamic acids is prepared. The tendency of diacylation can be minimized by employing one molar equivalent of the acid chloride (Hearn et al., 1969). Newly two methods have been developed which converted the aldehyde to hydroxamic acid. The first method was the formation of hydroximoyl chloride (RC.Cl = NOH) (Chiang et al., 1971). Another method was the preparation of aluminium isopropoxide in the presence of aldehyde with nitrosobenzene (Neunhoeffer et al., 1961). The formation of N-phenylsalicylohydroxamic acid was reported by Ghosh and Bhattacharya (Ghosh et al., 1964) while studying the condensation of salicyloylchloride with β- phenylhydroxylamine by Bamberger method (Bamberger et al., 1903).

2-Substituted aminonicotinhydroxamic acid has been synthesized when substituted nicotinic acid was treated with NH_2OH.



R= tolyl, anisyl, xylyl, $Cl_2C_6H_3$

2-substituted aminonicotinohydroxamic acid

1.3. Bacteria

Bacteria are microscopic single-celled organisms that flourish in different environments. Among the prokaryotic microorganisms, bacteria are occupied a large domain. They can live inside the human gut. They are also survived in symbiotic and parasitic relationships with plants and animals. They are vital in many stages of the nutrient cycle by recycling nutrients such as the fixation of nitrogen from the atmosphere. Bacteria are responsible for the decomposition of dead bodies in the nutrient cycle (Forbes et al., 2008). The largest number of bacteria exists in the gut flora and on the skin (Sears et al., 2005). Some bacteria are pathogenic and cause infectious diseases including anthrax, leprosy, cholera, syphilis, and bubonic plague. Sometimes they provide a beneficial role e.g., production of antibiotics, different enzymes, organic acids vaccines and different probiotics for curdling of milk and boosting of immune system etc. They are unicellular microbes that have the following features in common:

- A single circular chromosome
- Prokaryotic (undefined nucleus)
- Ribosomes
- Pili (used in conjugation)

- A rigid cell wall (gives bacteria a clear shape)
- Reproduction by binary fission (mitosis)

1.3.1. Bacterial virulence

Bacteria can increase their virulence in three basic ways:

- To prevent identification by the host immune system forms a capsule.
- They produce enzymes that inhibit the inflammatory response.
- In adverse conditions form a spore to survive.

1.3.2. Antibacterial Agents

Infectious diseases are the major causes of human sickness and death. In mid of 19^{th} century antibiotics are the promising agents to overcome such health care issues. A subclass of antibiotics is known as antibacterial. In earlier antibacterial have been classified several ways; but nowadays, antibacterial agents are classified into five groups to make it easily understandable. They are based on the type of action, the spectrum of activity, source, function, and chemical structure.

Based on the type of action

Bacteriostatic antibacterials: Sulphonamides, chloramphenicol, Spectinomycin, Trimethoprim, Tigecycline, Erythromycin, azithromycin, Linezolid, Doxycycline, tetracycline.

Bactericidal antibacterials: Penicillins (penicillin G, methicillin, cloxacillin, dicloxacillin), carbapenems (imipenem, aztreonam, ticaracillinclvulnate), gentamycin, quinolones, vancomycin, polymyxins.

Based on the source of antibacterial agents

- *Natural antibiotics/antibacterials*: Cephalosporins, cephamycins, benzylpenicillin, and gentamicin
- *Semi-synthetic antibiotics*: Ampicillin and amikacin+
- *Synthetic antibiotics*: Moxifloxacin and norfloxacin

Based on the spectrum of activity

- *Broad-spectrum antibacterials:* Ampicillin, Cephalosporins, Carbapenems, Ticarcillin, Rifamycins, Aminoglycosides (kanamycin A, amikacin, tobramycin, gentamicin), Macrolides (erythromycin, roxithromycin, clarithromycin, azithromycin), Quinolones (lomefloxacin, ofloxacin, norfloxacin, gatifloxacin, ciprofloxacin)
- *Narrow-spectrum antibacterials:* Penicillin-G, Cephalosporins, Cloxacillin (dicloxacillin flucloxacillin), methicillin, nafcillin, Vancomycin, clindamycin.

Based on chemical structure

- *β-Lactams*: Penicillins, Cephalosporins, carbapenems
- *Aminoglycoside*: Streptomycin, gentamicin, sisomicin, netilmicin, kanamycin
- *Macrolides*: Erythromycin and roxithromycin
- *Nitrobenzene derivatives*: Chloramphenicol
- *Quinolones and fluoroquinolones*: Nalidixic acid (1^{st} generation), ciprofloxacin (2^{nd} generation), levofloxacin (3^{rd} generation), and trovafloxacin (4^{th} generation).
- *Streptogramin antibiotics*: Dalfopristin and quinupristin
- *Sulphonamides*: Sulfadiazine, sulfonylureas, and thiazide diuretics
- *Tetracyclines*: Oxytetracycline, doxycycline.
- *Nitroimidazoles*: Metronidazole, Tinidazole

- *Nitrofuran derivatives*: Nitrofurantoin, Furazolidone
- Polyene antibiotics: Nystatin, Amphotericin-B, Natamycin
- *Nicotinic acid derivatives*: Isoniazid, Pyrazinamide, Ethionamide
- *Miscellaneous*: Rifampicin, Clindamycin, Spectinomycin, Vancomycin, Ethambutol, Thiacetazone, Clofazimine, and Griseofulvin

Function-based classification of antibacterial drugs

- *Cell wall synthesis inhibitors*: Penicillins, Cephalosporins, Vancomycin.
- *Inhibitors of membrane function*: Polymyxin, Gramicidine, Tyrothricin
- *Inhibitors of protein synthesis*: Tetracyclines, Macrolides, Chloramphenicol, Clindamycin,
- *Inhibitors of folate-dependent pathways*: Sulphonamides.
- *DNA gyrase inhibitors*: Fluoroquinolones.

Based on the type of organism

- *Antibacterial*: Penicillins, Aminoglycosides, Erythromycin, Fluoroquinolones, etc.
- *Antifungal*: Griseofulvin, Amphotericin-B, Ketoconazole, etc.
- *Antiviral*: Idoxuridine, Acyclovir, Amantadine, Zidovudine etc.
- *Antiprotozoal*: Chloroquine, Pyrimethamine, Metronidazole, Diloxanide etc.
- *Anthelmintics*: Mebendazole, Piperazine, Pyrantel, Niclosamide etc.

Recent antimicrobial agents

Tigecycline, Doripenem, Retapamulin, Telavancin, Ceftaroline, Fidaxomicin

FDA approval awaiting antibacterial agents: Ceftobiprole, Iclaprim, Torizolid, Radezolid, Cethromycin, Solithromycin, Oritavancin, Dalbavancin.

1.4. Fungus

The fungus is eukaryotic organisms that absorb nutrients directly and digests food externally by its cell walls. In ecological systems, they are the principal decomposers. A large no of fungi emulates by spores and their body (thalamus) composed of microscopic tubular cells which are known as hyphae. As like animals, when fungi get their carbon and energy from other organisms they called heterotrophs and when they take their nutrients from a living host (plant or animal) they are called biotrophs, when obtaining their nutrients from dead plants or animals and are called saprotrophs (saprophytes, saprobes) and when they kill host cells in order to get their nutrients they are called necrotrophs.

1.4.1. Fungi associated with animals

Fungi are grown on both invertebrate and vertebrate animals. When fungi attack insects and nematodes they are called "entomopathogens," which include a wide range of fungi in phyla Chytridiomycota, Ascomycota and Zygomycota. Fungi infect and engulf insects like caterpillars and ants to make conspicuous stomata that come out from their victim's body (Evans et al., 2011). There are different types of fungal infections or "mycoses" were found in humans and most common is occurred by dermatophytes, means dead of keratinized tissue including skin, finger, and toenails. A superficial infection like ringworm is occurred by dermatophytes. It is a serious problem but very difficult to treat. Some fungi are found in the microflora in healthy people, but they become pathogenic when predisposing conditions are arrived like, in the mucosal tissues of many healthy people yeast infections is observed by Candida species are called as candidiasis. Some fungi are inhaled as spores and they spread infections through the lungs such as Coccidioides immitis (commonly known as valley fever), and Histoplasma capsulatum (histoplasmosis). Few fungal pathogens are normally not related to humans and other

animals but can cause serious infections in weakened or healthy individuals when inhaled or implanted in wounds they are called opportunistic fungus. Most important opportunist fungus Aspergillus fumigates can produce small, airborne spores that are recurrently inhaled. In some cases, fungus starts growing invasively, which can cause aspergillosis.

1.4.2. Classification of fungi

Fungi may be classified as Yeast and Moulds

- Yeasts: Blastomyces, candida, histoplasma, coccidioides, Cryptococcus.
- Molds: Aspergillus spp. Dermatophytes

Clinically classified as:

- Superficial mycosis and Deep (systemic) mycosis
- Systemic fungal infections: Systemic candidiasis, Cryptococcal meningitis, Coccidiodomycosis, endocarditis, Rhinocerebral mucormycosis, Histoplasmosis (cough, fever, multiple pneumonics infiltrates), Pulmonary aspergillosis, Blastomycosis (pneumonitis, with dissemination), Pneumocystis carinii pneumonia.

Classification based on mechanism of action

- Fungal cell wall synthesis inhibition: Caspofungin.
- Bind to fungal cell membrane ergosterol: Amphotericin–B, Nystatin.
- Inhibition of ergosterol + lanosterol synthesis: Terbinafine, Naftifine, Butenafine.
- Inhibition of ergosterol synthesis: Azoles
- Inhibition of nucleic acid synthesis: 5–Flucytosine.
- Disruption of mitotic spindle and inhibition of fungal mitosis: Griseofulvin.
- Miscellaneous: • Ciclopirox, Tolnaftate, Haloprogin, Undecylenic acid, Topical azoles.

Classification based on the structure

1. Antibiotics
   - Polyene: Amphotericin, nystatin, hamycin
   - Heterocyclic benzofuran: griseofulvin
2. Antimetabolite: Flucytosine
3. Azoles
   - Imidazoles: Ketoconazole, clotrimazole, oxiconazole, miconazole,
   - Triazoles: Fluconazole, itraconazole, voriconazole,
4. Allylamines – Terbinafine, butenafine
5. Echinocandins – Caspofungin, anidulafungin, micafungin
6. Other topical agents – Tolnaftate, Undecyclinic acid, benzoic acid

1.4.3. Triazole as an antifungal

Imidazoles or triazoles are azole group of antifungals that possess two or three nitrogen atoms in their azole ring respectively. Triazoles are any heterocyclic compound, having a five-member ring of two carbon atoms and three nitrogen atoms. Triazole has two types of isomers1,2,3-Triazole and 1,2,4-Triazole that differ in the relative positions of the three nitrogen atoms. They have two tautomers that differ by which nitrogen has a hydrogen bonded to it. Imidazoles have no role for inhibition on *Aspergillus* species and thus they are predominately used to treat superficial and mucosal infections (Pasqualotto AC., 2010). Naturally, fungi are eukaryotes and share many of the same potential drug receptor targets with humans and other mammals. For this reason, the design of potent antifungal that is highly selective for fungal receptors and weakly attracted to human or mammalian receptors has been the goal of pharmaceutical companies for many years. Amphotericin B is the first commercially available broad-spectrum antifungal for

the treatment of systemic fungal infections, but it has a difficult safety profile. It causes sometimes a chronic renal failure in patients due to its nephrotoxicity if taken more than 4 g. Despite the difficulties of evaluating fungi *in vitro* and *in vivo*, there have been slow advances in the development of highly selective antifungal agents, particularly the azole class. Due to their spectrum of activity, pharmacokinetic properties and relatively low frequency of adverse events compared with amphotericin B, azole antifungals have made their way into the daily arsenal of medical professionals, battling various types of invasive fungal infections (Pasqualotto AC., 2010).

Table 1: Potential drug interactions encountered with triazole antifungal drugs (Lass-Flörl C., 2011)

Drug	Fluconazole	Itraconazole	Voriconazole	Posaconazole
Ciclosporin	++	++	+++	++
Sirolimus	++	++	++++	++
Tacrolimus	++	++	+++	++
Calcium channel blockers	++	++	++	++
Busulfan	None	++	++	
Vinca alkaloids	++	++	++	++
Midazolam	↑fluconazole	++	++	++
Simvastatin	+	++++	++++	+++
Omeprazole	None ↓	↓Itraconazole	↑Voriconazole	↑Posaconazole

+ indicates mild effects; ++ indicates moderate effects; +++ indicates high effects; ++++ indicates very high effects; ↓indicates decreased plasma concentrations; ↑indicates increased plasma concentrations.

1.4.4. *In Vitro* and *In Vivo* Antifungal Activities

In the case of *Candida krusei, Aspergillus spp., Fusarium spp., Scedosporium spp.* or zygomycetes and some strains of *Candida glabrata* fluconazole has no activity (Lossen et al., 1873). Itraconazole has potent activity against the majority of *Candida spp*. and *Aspergillus spp*. but restricted activity against zygomycetes. Voriconazole has activity against *Candida krusei* (Chauhan et al., 2016) Posaconazole has the broadest spectrum activity against *Candida spp*., *Aspergillus spp*. and many zygomycetes (Lossen et al., 1873).

1.4.4.1. Invasive Fungal Infections

The most invasive fungal infection is candidiasis caused by *C. albicans* (40–50% candidaemias). This type of infection arises due to azole-resistant *Candida* spp. In neutropenic and organ-transplant patient *A. fumigatus* and other *Aspergillus spp*. are common pathogenic molds and cause major morbidity (Perkhofer et al., 2010). Other molds, like *Scedosporium* spp. and the *Zygomycetes* are caused high death depending on the site of infection and host factors. In antifungal therapy because of drug resistance for fungal pathogens, the accurate identification of the pathogen is critical. Fluconazole and itraconazole are the common fungi used for the infection that occurred by *Aspergillus spp* by penetrating infection in neutropenic and transplant patients during antifungal prophylaxis and voriconazole, azole-resistant *Candida spp*. (Lass-Flörl C., 2009).

1.4.4.2. Fluconazole and Invasive Candidiasis

Fluconazole is the first-generation triazole with a molecular weight of 306.271 g/mol. Fluconazole is derived from ketoconazole and it is active in animal models for combating fungal infections. In comparison to other triazole fluconazole has limited activity. Fluconazole was developed for the treatment of systemic and superficial fungal infections (Monif GR., 1999). It is

highly active against yeast but inactive against filamentous fungal infections. Fluconazole is available in tablet, capsule, powder or injectable forms. Gafter-Gvili et al. (Gafter-Gvili et al., 2008) performed fifteen randomized controlled trials of different antifungal agents for the treatment of candidaemia and invasive candidiasis. Fluconazole compared with other drugs like amphotericin B deoxycholate, itraconazole or a combination of fluconazole and amphotericin B deoxycholate (nine comparisons), four comparing echinocandins with other drugs (fluconazole, amphotericin B deoxycholate and liposomal amphotericin B), one comparing micafungin and caspofungin, and one comparing amphotericin B deoxycholate plus fluconazole and voriconazole. All the antifungal agents have similar efficacy, but the microbiological failure was higher with fluconazole than with amphotericin B or anidulafungin. Amphotericin B caused a higher adverse effect than fluconazole. In general, triazoles are fungistatic but some have fungicidal activity in particular fungi, like voriconazole or itraconazole against Aspergillus spp.

1.5. Cancer

Cancer is a group of more than 100 different diseases in which abnormal cells divide without control and can invade nearby tissues. Cancer cells can also spread to other parts of the body through the blood and lymph systems. Possible signs and symptoms are abnormal bleeding, a lump, weight loss, prolonged cough and a change in bowel movements. These symptoms may also indicate other causes (Cancer – Signs and symptoms, 2014) There are several main types of cancer. Carcinoma is cancer that begins in the skin or in tissues that line or cover internal organs. Sarcoma is cancer that occurs in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is cancer that starts in blood-forming tissue, such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

Lymphoma and multiple myeloma are cancers that happen in the cells of the immune system. Central nervous system cancers are cancers that begin in the tissues of the brain and spinal cord.

1.5.1. Most common types

- Breast cancer: Cancer that forms in the cells of the breasts.
- Prostate cancer: Cancer in a man's prostate, a small walnut-sized gland that produces seminal fluid.
- Basal cell cancer: A type of skin cancer that begins in the basal cells.
- Melanoma: The most serious type of skin cancer.
- Colon cancer: A cancer of the colon or rectum, located at the digestive tracts lower end.
- Lung cancer: Cancer that begins in the lungs and most often occurs in people who smoke.
- Leukemia: A cancer of blood-forming tissues, hindering the body's ability to fight infection.
- Lymphoma: A cancer of the lymphatic system.

1.5.2. Key features of cancer

- Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths, WHO, 2008).
- The most frequent types of cancer differ between men and women.
- Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year.
- About 30% of cancer deaths are due to the five leading behavioral and dietary risks; high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use.

- Tobacco use is the most important risk factor for cancer causing 22% of global cancer deaths and 71% of global lung cancer deaths.
- Cancer caused by viral infections such as HBV/HCV and HPV is responsible for up to 20% of cancer deaths in low and middle-income countries.
- Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 (WHO, 2008).

1.5.3. Difference between benign and malignant cancer

Tumors can be benign (noncancerous) or malignant (cancerous). Benign tumors have a tendency to grow slowly and do not spread. Malignant tumors can grow rapidly, invade and destroy nearby normal tissues, and spread throughout the body.

1.5.3.1. Locally invasive cancer

The tumor can attack the tissues surrounding it by sending out "fingers" of cancerous cells into the normal tissue.

1.5.3.2. Metastatic cancer

The tumor can send cells into other tissues in the body, which may be distant from the original tumor.

1.5.3.3. Primary tumors

The original tumor is called the "primary tumor." Its cells, which travel through the body, can begin the formation of new tumors in other organs. These new tumors are referred to as primary tumors.

1.5.3.4. Secondary tumors

The cancerous cells travel through the blood (circulatory system) or lymphatic system to form secondary tumors. The lymphatic system is a series of small vessels that collect waste from cells,

carrying it into larger vessels, and finally into lymph nodes. Lymph fluid eventually drains into the bloodstream.

1.5.4. Cause of Cancer

Cancer is a complex group of diseases with many possible causes. There is no one single cause for cancer. Scientists believe that it is the interaction of many factors together that produces cancer. The factors involved may be genetic, environmental, or constitutional characteristics of the individual.

Diagnosis, treatment, and prognosis for childhood cancers are different than for adult cancers. The main differences are the survival rate and the cause of cancer. The overall five-year survival rate for childhood cancer is about 80%, while in adult cancers the survival rate is 68%. This difference is thought to be because childhood cancer is more responsive to therapy and a child can tolerate more aggressive therapy.

Childhood cancers often occur or begin in the stem cells, which are simple cells capable of producing other types of specialized cells that the body needs. A sporadic (occurs by chance) cell change or mutation is usually what causes childhood cancer. In adults, the type of cell that becomes cancerous is usually an epithelial cell. Epithelial cells line the body cavity and cover the body surface. Cancer occurs from environmental exposures to these cells over time. Adult cancers are sometimes referred to as acquired for this reason.

1.5.5. Signs and symptoms of cancer

- A change in the size, shape or color of a mole
- A cough or croaky voice that lasts longer than three weeks
- Persistent difficulty swallowing or indigestion
- A sore that won’t heal after several weeks

- A mouth or tongue ulcer that lasts longer than three weeks
- Problems passing urine
- Blood in urine
- Blood in bowel motions
- A change to more frequent bowel motions that lasts longer than four to six weeks
- Unexplained weight loss or heavy night sweats
- An unexplained pain or ache that lasts longer than four weeks
- Breathlessness
- Coughing up blood

1.5.6. Classification of Anticancer Drugs

***Polyfunctional alkylating agents*:** Nitrosoureas, Nitrogen mustards, Busulphan, Ethylenimines

***Other Alkylating Drugs*:** Procarbazine, Dacarbazine, Cisplatin

***Antimetabolites*:** Methotrexate

***Purine antagonists*:** Mercaptopurine (6-MP), Thioguanine (6-TG), Fludarabine Phosphate

***Pyrimidine antagonists*:** Fluorouracil (5-FU), Cytarabine, Azacitidine

***Plant alkaloids*:** Vinblastine, Vincristine, Etoposide, Teniposide, Paclitaxel, Docetaxel

***Antibiotics*:** Anthracyclines, Doxorubicin, Daunorubicin, Dactinomycin, Idarubicin, Mithramycin, Mitomycin, Bleomycin

***Monoclonal Antibodies*:** Alemtuzumab, Bevacizumab, Cetuximab

***Hormonal agents*:** Tamoxifen, Flutamide, Leuprolide, Aminoglutethimide,

***Miscellaneous anticancer drugs*:** Amsacrine, Hydroxyurea, Asparaginase, Mitoxantrone, Mitotane, Amifostine

***Other measures*:** Radiotherapy**,** external radiotherapy**,** internal radiotherapy**,** surgery**,** Laser treatment**,** Photodynamic therapy**.**

1.5.7. Triazole as anticancer agents

1,2,4 triazoles have a wide range of pharmacological activities (Sztanke et al., 2008) such as antimicrobial (Ikizler et al., 1999) and antitumor (Demirbas et al., 2004) properties. The other ones show also anti-inflammatory (Tozkoparan et al., 2000), antihypertensive (Emilsson et al., 1985), anticonvulsant and antiviral (Kritsanida et al., 2002), analgesic (Turan-Zitouni et al., 1999) activities. 1,2,4 triazole derivatives are also found in different type of medicine like fluconazole, itraconazole (azole antifungal agents), estazolam (hypnotic, sedative), etoperidone (antidepressant), alprazolam (tranquilizer), rilmazafon (hypnotic, anxiolytic, used in the case of neurotic insomnia), benatradin (diuretic), trapidil (hypotensive), nefazodone (antidepressant, 5-HT2 A-antagonist), anastrozole, letrozole, vorozole (antineoplastics, nonsteroidal competitive aromatase inhibitors).

1.5.8. Hydroxamic acid as anticancer agents

According to the World Health Organization (WHO), cancer is one of the most severe problems around the world (Hematpoor et al., 2018). Breast cancer is one of the most important causes of cancer death among women worldwide. Tamoxifen and Toremifene are used for the chemotherapy in breast cancer patient but the drug resistance and severe side effects major problems in clinical oncology (Bachmeier et al., 2011). That's why there is a need for the search of novel anti-cancer compounds. Among all the breast cancer cell lines estrogen non-dependant MDA-MB-231 is one of the most extensively used models (Chavez et al., 2010). Hydroxamic acids or hydroxamates are carboxylic acids or aldehyde analogs where -COOH group has been replaced by -CONHOH or -CHO group has been replaced by -CONHR (Kokare et al., 2008).

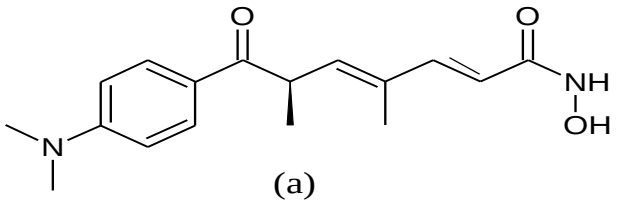
Hydroxamic acids are successful molecules in the field of cancer chemotherapy. SAHA (Marks et al., 2007 and Kumar et al., 2015), PXD-101 (Belinostat,Topotarget) (Foss et al., 2015) and LBH-589 (panobinostat) (Atadja et al., 2009) are the hydroxamates based drugs for cancer chemotherapy, approved by the U.S. Food and Drug Administration (FDA) in October 2006, July 2014 and February 2015, respectively. Some other hydroxamate-based molecules are also in clinical trials, like m-carboxycinnamic acid bishydroxamic acid (CBHA) (Song et al., 2014), SB-939 (phase II) (Eigl et al., 2015) and 4SC-201 (Resminostat, phase II) (Brunetto et al., 2013). SAHA (Suberoylanilide Hydroxamic Acid) is considered as a potent anticancer agent among all hydroxamic acid derivatives (Paris et al., 2008) because of their very good chelating capability (Zhao et al., 2013), histone deacetylase (HDAC)which is advantageous for enzyme inhibition and thus have power over a crucial role in cancer drug discovery. That's why the hydroxamates group grabs the attraction for scientists from all over the world.

1.6. Histone Deacetylase Inhibitors (HDACi)

For the last four decades, a number of potential approaches have been proposed for the treatment of cancer. One of the recent targets is Histone deacetylase (HDAC). Inhibition of histone deacetylases (HDACs) is emerging as a new strategy in human cancer therapy. Acetylation of nuclear histones plays a crucial role in gene expression since histone hyperacetylation or hypoacetylation has been found to be clearly associated with transcriptionally activated or inactive genes. Enzymatic complexes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) have been identified as transcriptional coactivators and transcriptional corepressors, respectively. Modification of histone acetylation level, promoted by HAT and HDAC enzymes, has been recognized to play an important role in the epigenetic modulation of gene expression; in fact this well-known post-translational mechanism is highly involved in the

modulation of chromatin plasticity and in the regulation of transcriptional factors accessibility to DNA (Biel et al., 2005); therefore the disruption of histone acetylation pattern is supposed to determine transcriptional disorders and is related to several malignant diseases (Momparler et al., 2003). The inhibition of HDAC enzyme has proven to induce anti-proliferative effects and to promote cellular differentiation. For these reasons, the discovery of new agents targeting HDAC enzyme is considered of great interest for the development of anticancer drugs (Bolden et al., 2006). Histone deacetylase inhibitors (HDACIs) are novel and promising antitumor agents. HDACIs can activate the transcription of specific genes through the accumulation of histone acetylation, subsequently leading to a variety of phenotypic changes, such as cell cycle arrest, differentiation and apoptosis (Cohen et al., 1999 and Finzer et al, 2001]. Specific triazole-linked cap group substitution patterns and spacer-group chain lengths that enhance the antimalarial and antileishmanial activity of aryltriazolylhydroxamates-based HDACI (Patil et al., 2010) also.

The classic pharmacophore for HDAC inhibitors consists of three distinct structural motifs: the zinc-binding group (ZBG), a hydrophobic linker, a connecting unit and a recognition cap group **(Fig. 4)**. The X-ray structures of a bacterial HDAC homolog, histone deacetylase-like protein (HDLP), bound to SAHA or Trichostatin A (TSA) [a] and, more recently,



(a)

human HDAC8 confirmed that the ZBG interacts with a Zn^{2+} ion at the base of a channel-like active site.

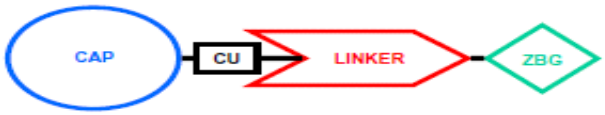


Fig 4. HDACIs common pharmacophore schematic segmentation

In a prototypical HDAC inhibitor, the cap group could be linked to the aliphatic linker group through either hydrogen-bond accepting or donating groups such as keto- and amide-groups. The apparent lack of preference for H-bond donor or acceptor in the linking-moiety presents an opportunity to incorporate other more synthetically accessible and pharmacokinetically desirable moieties that may help simplify the molecular design and synthesis of novel HDAC inhibitors. We proposed that 1, 2, 4-triazole ring could act as a linking moiety which joins the cap group to the linker group in a HDAC inhibitor.

The triazole ring will serve two purposes:

- It could facilitate stronger cap group interactions with the amino acid side chains at the entrance of the HDAC active site;
- It could serve as an isostere to the pharmacokinetically and toxicologically disadvantageous groups such as amide and ketone.

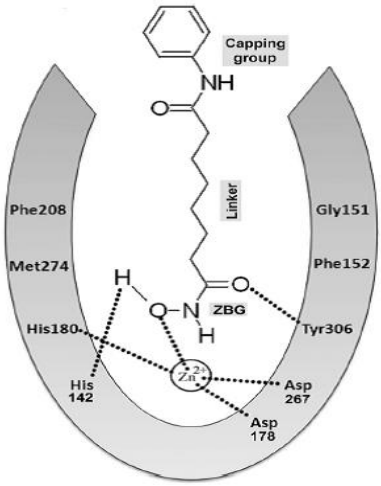


Fig 5: Schematic representation of basic pharmacophore model and binding of hydroxamic acid based HDACi

The hydrophobic linker efficiently presents the ZBG to the active site by filling the channel while the cap group at the other end of the linker makes contacts with amino acid residues at the rim of the channel. The common ZBG of HDAC inhibitors is the hydroxamate moiety. The structural modifications of the hydroxamate ZBG have been modestly successful; yielding isosteres such as benzamide, a-ketoesters, electrophilic ketones, mercaptoamide and phosphonates.

1.7. Leishmaniasis

Leishmaniasis is a vector-borne disease caused by flagellated protozoans of the genus Leishmania. The disease is widespread in the tropical and subtropical areas and found in 98 countries in Europe, Africa, Asia and America (Alvar et al., 2012). However, over 90% of new cases occur in just 13 countries (Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Columbia, Ethiopia, India, Iran, Peru, South Sudan, Sudan and Syria). It is estimated that between 0.9 and 1.7 million people are newly infected every year, but only a small fraction of them will develop the disease and 20,000–30,000 will eventually die. In prehistorical times the existence of

Leishmania-like species is recognized in two fossil ambers. The first Leishmania-like fossil was found in the proboscis and alimentary tract of a blood-filled female of the extinct sand fly Palaeomyia bursitis preserved in 100 million-year-old Cretaceous Burmese amber (Poinar et al., 2004). The Leishmania-like species was described in a new, collective fossil genus Paleoleishmania and named P. proteus (Poinar et al., 2004). Alongside promastigotes and paramastigotes, amastigotes were also found indicating that the sand fly acquired the parasite from the blood of a vertebrate during feeding (Poinar et al., 2004). The presence of amastigotes is suggestive of a digenetic life-cycle of P. proteus. The blood cells were subsequently identified as being of a reptile (Poinar et al., 2004). The second Leishmania-like fossil was known as Paleoleishmania neotropicum and was found in the extinct sand fly Lutzomyia adiketis in a 20–30 million-year-old Dominican amber (Poinar et al., 2008). Promastigotes, paramastigotes and amastigotes were observed in the gut and proboscis of the sand fly; however, no vertebrate blood cells were found (Poinar et al., 2008). Nevertheless, the presence of amastigotes and the fact that no monogenetic flagellates colonize sand flies suggest a digenetic life-cycle of P. neotropicum with a vertebrate host. This fossil record also provides evidence that Neotropical sand flies were vectors for Leishmania-like parasites in the mid-Oligocene to early-Miocene. Johnson defined the taxonomy in the year of 1974 (Johnson et al., 1974) as "The science of placing a biological form in order" and that science was started with the work of Linnaeus in the 18th century. Van Leeuwenhoek was the first person who observed protozoa but then he didn't include that simple organism into his classification but in 1845 Von Siebold was the first man who defined the term 'Protozoa'. After 40 years in 1885 Cunningham, a medical officer was first observed leishmanial amastigotes inside the macrophages from 'Delhi boil' in India but still, he didn't name it (Cunningham et al., 1885). In 1900 Leishman found amastigotes in a patient infected with

visceral leishmaniasis in Calcutta and in 1903 he described it. In the same year after Leishman, Donovan also reported in Madras. Later in 1903 Ross named the parasite, Leishmania. Later in the same year in Boston, Wright found amastigotes from a biological sample withdrawn from a child came from Armenia with cutaneous lesion and he named it *Helicisima tropicum* (Wright et al., 1903), but in 1906 Luhe named it as *Leishmania tropica* (Lühe et al., 1906). In the year of 1908, in the Mediterranean, Nicolle found the same parasite in an infant suffering from kala-azar, he named it *Leishmania infantum* (Nicolle et al., 1908) Similarly three years later Vianna named the parasite *Leishmania brazilienses,* found in a patient with cutaneous lesion in Brazil (Vianna G., 1911). And so on the different species of the same genus leishmania were named by scientists across the world. Depends on the clinical manifestations there are mainly three types of leishmaniasis: Visceral leishmaniasis (VL), Cutaneous leishmaniasis (CL) and Mucocutaneous leishmaniasis (MCL).

1.7.1. Visceral leishmaniasis (VL)

VL is also known as kala-azar in the Indian sub-continent. It is caused by the protozoan parasites *Leishmania donovani* and *Leishmania infantum* (*Leishmania chagasic*), and is a potentially fatal disease with a worldwide distribution, in Asia, East Africa, South America, and the Mediterranean region. The parasites are transmitted through the bite of female phlebotomine sand flies and in the human host are obligate intracellular parasites of the reticuloendothelial system, surviving and multiplying in different macrophage populations. In patients it is diagnosed by anemia, splenomegaly, pancytopenia, irregular fevers, weight loss and weakness occurring progressively over a period of weeks or even months. Almost all clinically symptomatic (non-immune) patients die within months if untreated. Sub-clinical infection in partially immune human carriers may be an important reservoir of infection. Other mammals,

often canids, either domesticated or wild, act as an additional zoonotic reservoir of infection, especially of *L. infantum*.

VL occurs poor, remote, and in certain politically unstable areas, where there is limited health care and patients have little access to affordable medications and it is estimated that every year about 90% of the 50,000 to 90,000 new cases arising in the rural areas of India, Sudan, South Sudan, Kenya, Somalia, Ethiopia, and Brazil whereas the cases of kala-azar is decreasing in the three major endemic countries like India, Nepal, and Bangladesh. However, still, now the disease shows a high fatality rate. It gets more complicated when recovered patients develop post kala-azar dermal leishmaniasis.

1.7.2. Cutaneous leishmaniasis (CL)

CL is not life-threatening but it is the most common form of leishmaniasis affecting humans. It causes skin lesions, mainly skin ulcers. It occurs generally on exposed body parts, which may leave life-long scars and sometimes serious disability. It can become disseminated and produce generalized debilitating disease in immunosuppressed persons (e.g. HIV-affected patients). The exact cause of cutaneous leishmaniasis is not known. Different forms of cutaneous leishmaniasis are caused by over 15 different species of the protozoan parasite *Leishmania*, transmitted by infected female sandflies. The reservoir host is either infected humans (anthroponotic CL-ACL, post kala-azar dermal leishmaniasis – PKDL) or mammals (zoonotic – ZCL). Anthroponotic CL is caused by L. tropica and is common in Afghanistan, Iran, Iraq, Jordan, Lebanon, Syria, Turkey, and parts of Saudi Arabia and India. ACL tends to heal spontaneously within 1-2 years but leaves disfiguring scars. Zoonotic CL is widespread in Central and South America and caused by two subgenera of the parasite: *Leishmania leishmania* (e.g., *Leishmania*

mexicana, *Leishmania amazonensis*) and *Leishmania viannia* (e.g., *Leishmania braziliensis*, *Leishmania guyanensis*).

1.7.3. Mucocutaneous leishmaniasis (MCL)

MCL cases are focused in South America, especially in Brazil, Paraguay, Ecuador, Bolivia, Peru, Colombia, and Venezuela. Ninety percent of the cases occur in Brazil, Bolivia, and Peru. Twenty percent of leishmaniasis patients in Brazil develop MCL. In Ecuador, many of the cases seem to be focused in the Amazon region. During the 1990-2003 period, there were 21,805 reports of MCL mostly from the Amazonian lowlands, some inter-Andean valleys, and throughout the Pacific coastal region. Other infections caused by various Leishmania species have occurred in Ethiopia, Kenya, Namibia, Central America, Guyana, Surinam, Panam, and Sudan. Although the pathogenesis of visceral and cutaneous leishmaniasis are well understood, the pathogenesis of MCL is still unclear. MCL causes partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. However, it is believed that host genetic factors are important in the advancement of the disease. MCL development is similar to that of cutaneous leishmaniasis, and the two infections can occur simultaneously. MCL occurs when cutaneous lesions expand to the mucosal region or through metastasis. Moreover, it is not uncommon for MCL to develop many years after the recovery of an initial lesion. The result is a gradual and progressive development of destructive lesions.

1.7.4. Post-kala-azar dermal leishmaniasis (PKDL)

PKDL is a complication of VL in areas where *Leishmania donovani* is endemic; it is characterized by a hypopigmented macular, maculopapular, and nodular rash usually in patients who have recovered from VL. It usually appears 6 months to 1 or more years after the apparent cure of the disease but may occur earlier or even concurrently with visceral leishmaniasis

especially in Sudan. PKDL heals spontaneously in the majority of cases in Africa but rarely in patients in India. It is considered to have an important role in maintaining and contributing to the transmission of the disease particularly in interepidemic periods of VL, acting as a reservoir for parasites.

Table 2. Status of endemicity of cutaneous leishmaniasis and visceral leishmaniasis at the year of 2015 (WHO data)

	Status of endemicity		**Number of cases reported**	
Country	**Cutaneous leishmaniasis**	**Visceral leishmaniasis**	**Cutaneous leishmaniasis**	**Visceral leishmaniasis**
United States of America	Endemic	No cases reported	No data	No cases reported
Mexico	Endemic	Endemic	479	1
Venezuela	Endemic	Endemic	2013	37
Colombia	Endemic	Endemic	7541	21
Ecuador	Endemic	No cases reported	No data	No cases reported
Peru	Endemic	No cases reported	5459	No cases reported
Bolivia	Endemic	Endemic	2231	0
Paraguay	Endemic	Endemic	126	92
Argentina	Endemic	Endemic	336	8
France	Endemic	Endemic	No data	No data

Italy	Endemic	Endemic	No data	No data
Spain	Endemic	Endemic	No data	No data
Portugal	Endemic	Endemic	0	0
Slovenia	Endemic	Endemic	No data	No data
Bosnia and Herzegovina	Endemic	Endemic	No data	No data
Bulgaria	Endemic	Endemic	No data	No data
Greece	Endemic	Endemic	No data	No data
Morocco	Endemic	Endemic	2630	85
Mauritania	Endemic	Endemic	No data	No data
Algeria	Endemic	Endemic	7523	38
Tunisia	Endemic	Endemic	6741	31
Libya	Endemic	Endemic	No data	No data
Egypt	Endemic	Endemic	2043	0
Senegal	Endemic	Endemic	No data	No data
Guinea	Endemic	No cases reported	No data	No cases reported
Cote d'Ivoire	Endemic	Endemic	No data	No data
Ghana	Endemic	No cases reported	No data	No cases reported
Burkina Faso	Endemic	No cases reported	No data	No cases reported
Niger	Endemic	Endemic	No data	No data

Nigeria	Endemic	Previously reported cases	0	0
Chad	Endemic	Endemic	No data	No data
Sudan	Endemic	Endemic	No cases reported	2622
Cameron	Endemic	Previously reported cases	No data	No data
Republic of Congo	Endemic	Endemic	No data	No data
Ethiopia	Endemic	Endemic	301	1990
Kenya	Endemic	Endemic	160	762
Namibia	Endemic	No cases reported	No data	No cases reported
Turkey	Endemic	Endemic	No data	No data
Syrian Arab Republic	Endemic	Endemic	50972	20
Jordan	Endemic	Endemic	257	1
Iraq	Endemic	Endemic	18884	427
Saudi Arabia	Endemic	Endemic	No data	No data
Yemen	Endemic	Endemic	4063	5
Oman	Endemic	Endemic	0	1
Azerbaijan	Endemic	Endemic	18	28
Iran	Endemic	Endemic	18607	68

Kazakhstan	Endemic	Endemic	No data	No data
Uzbekistan	Endemic	Endemic	508	34
Afghanistan	Endemic	Endemic	19707	8
Pakistan	Endemic	Endemic	No data	No data
India	Endemic	Endemic	No data	8500
China	Endemic	Endemic	No data	No data
Thailand	Endemic	Endemic	1	1

1.7.5. Morphology and life cycle

Leishmania spp. is digenetic or heterogeneous parasites, whose life cycle involves two hosts, a vertebrate and an invertebrate, the sandfly. In *Leishmania*, the life cycle stage in the vertebrate is the amastigote and in the insect, the promastigote. During its life cycle *Leishmania* exist in two basic body forms: the amastigote, the intracellular form in the vertebrate host, and the promastigote, the extracellular form in the sandfly (*Phlebotomus* spp. and *Lutzomyia* spp.) vector. It was found that through the transformation from amastigote to promastigote, lengthening and elaboration of the mitochondrion (Creemers et al., 1967) took place, but in few cases, long and tortuous mitochondrion was observed in amastigotes (Pham et al., 1970). Amastigotes are taken up from the blood of an infected host when the female sandfly bites, and in the sandfly gut they develop into promastigotes where they multiply by binary fission; promastigotes move anteriorly into the proboscis and are introduced into the vertebrate host when the sandfly bites again. The promastigotes injected by the sandfly during feeding are phagocytized and develop into intracellular amastigotes.

The amastigote, literally "without a flagellum," is the intracellular, non-motile form in the vertebrate host, and it divides by longitudinal binary fission at 37ºC. Intracellular amastigotes are 3-6 um in length and 1.5-3.0 um in width. The amastigote is also called the Leishman-Donovan (LD) body. The amastigote is not really devoid of a flagellum, it is simply that the flagellum does not protrude beyond the body surface and by light microscopy cannot be seen. The promastigote, literally the body form with "an anterior flagellum" is 15-30 um in body length and 5mm in width; it is extracellular, motile, and grows and divides by longitudinal binary fission at 27˚C in the sandfly. Promastigotes can be grown in vitro at 25˚C temperature on the NNN medium, which has a solid phase of blood agar and a liquid phase containing a physiologic salt solution. Liquid media that support promastigote growth are also available. Amastigotes usually are grown inside tissue culture cells and can also be grown extracellularly at 37ºC under special conditions (Bray et al., 1969).

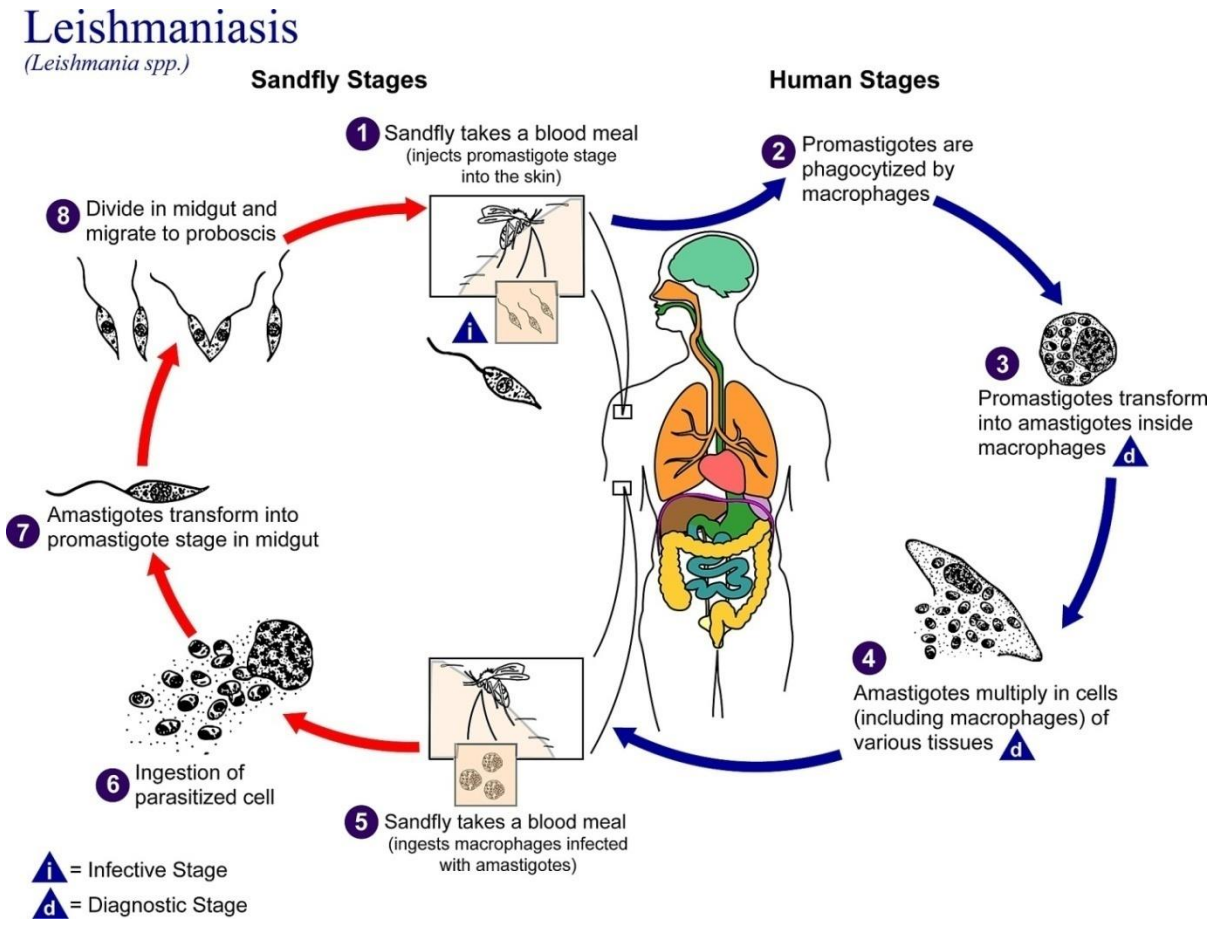


Fig 6. The life cycle of Leishmania parasite [From google]

1.8. Oxidation

Oxidation processes are intrinsic to the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms (Ślusarczyk et al., 2009). Free radicals are chemical species containing one or more unpaired electrons in their outermost shell, most of them being unstable and capable of abstracting electrons from other molecules (Dakubo et al., 2010). They are constantly formed in the human body but when generated in excess or in the presence of a deficiency in the naturally occurring antioxidant defenses it becomes toxic. If the amount of free radicals is increased it damages biomolecules like enzymes, lipids, proteins, and DNA in cells and tissues. As a result cancer, diabetes, cardiovascular and autoimmune

diseases, and neurodegenerative disorders, aging, and other diseases occurred through the aggressive reactivity of the free radicals (Torreggiani et al., 2005; Karalı et al., 2010 and Patil et al., 2013]. Antioxidants are very important compounds that reduce or neutralize free radicals, therefore keeping the cells from oxidative injuries. Antioxidants get interested for the treatment of huge number diseases like cancer, diabetes, aging, cardiovascular and other degenerative diseases. Different synthetic methodologies were designed for the development of some new antioxidants. A number of established methods are already available such as reducing the power method, superoxide radical scavenging, microsomal NADPH-dependent inhibition of lipid peroxidation (LP), microsomal ethoxyresorufin O-deethylase (EROD) activity, hydroxyl radical scavenging, nitric oxide radical scavenging, xanthine oxidase, cytochrome C, etc. DPPH (2,2, diphenyl-1-picrylhydrazyl) method is very common for the determination of antioxidant activity and has been found to be the most appropriate method (Kus et al., 2004).

1.8.1. Triazole as an antioxidant

Nowadays nitrogen-containing heterocyclic compounds such as triazole are getting more interesting topics in the medicinal chemistry. Triazole nucleus containing potent molecules such as Ribavirin, Rizatriptan, Alprazolam, Fluconazole and Itraconazole are currently used for the treatment of viral, migraine, anxiolytic and fungal diseases. (Knabe et al., 1983 and Lopes et al., 2004). The 1,2,4-Triazoles is an isomer of triazole and it is an interesting class of heterocyclic compound with a broad spectrum of pharmacological activities like antibacterial, antifungal (Karabasanagouda et al., 2007), antitumor (Ibrahim et al., 2009), anti-inflammatory, analgesic (Chawla et al., 2012), anthelmintic (El-Khawass et al., 1989), antihypertensive, antidepressant, anticonvulsant and antiviral (Kritsanida et al., 2002).

1.8.2. Hydroxamic acids as an antioxidant

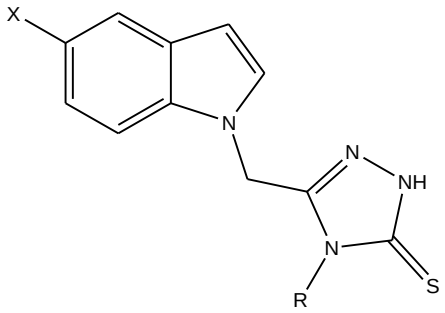
Hydroxamic acid (R-CO-NH-OH) is an important family of organic bioligands which is a derivative of hydroxylamine (NH_2OH) and carboxylic acids (R-COOH). Chemically, hydroxamic acid and its derivatives donate nitric oxide by way of their chemical reactivity. They have the ability to chelate metal ion and releasing nitric oxide (Arora et al., 2012). Its ability to delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions suggests them as potential antioxidants (Singh et al., 2014). They react with single free radicals and produce neutral molecules due to its ability to donate electrons and as potential antioxidants; they may be able to prevent cell and tissue damage as they act as a scavenger (Brandhuber et al., 2013).

Chapter 2

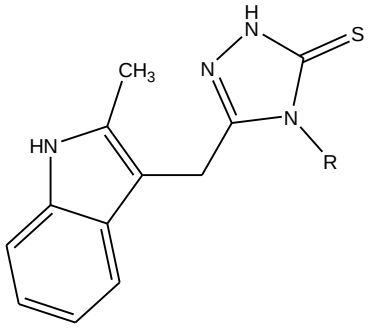
2. Literature Review

2.1. Antibacterial activity

Several 1,2,4-triazole linked indole containing derivatives were synthesized and screened for their antibacterial activity against four human pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* (Varvaresou et al., 2000). Some of them showed potential antibacterial activities. 3-[(5-Bromo-1H- 1-indolyl)methyl]-4-a-naphthyl-4,5-dihydro-1H-1,2,4-triazole-5- thione, (1) (X: Br, R: α-naphthyl) was active against *S. aureus*. They also reported that α-naphthyl substituted compound (2) (R: α-naphthyl) inhibited both *B. subtilis* and *E. coli* and compound (2) having only the phenyl (R: phenyl) was selectively active against *E. coli*.

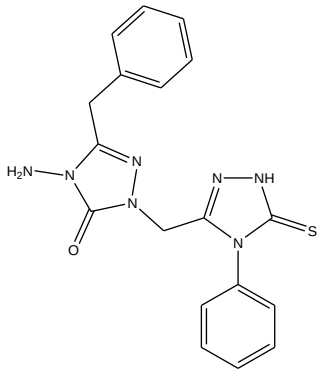


(1)

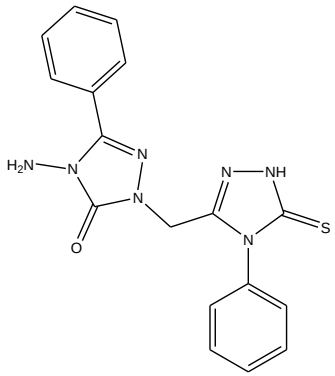


(2)

Demirbaş et al. reported that 1-(4-phenyl-5-thioxo-1,2,4- triazol-3-yl)methyl-4-amino-3-benzyl-5-oxo-4,5-dihydro-1,2,4- triazole (3) showed antibacterial activity against *S. aureus*. The inhibitory effect on mycelial growth by 1-(4-phenyl-5-thioxo- 1,2,4-triazol-3-yl)methyl-4-amino-5-oxo-3-phenyl-4,5-dihydro- 1,2,4-triazole (4) was observed (Demirbas et al., 2004).

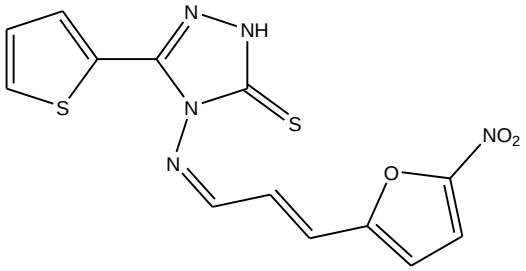


(3)



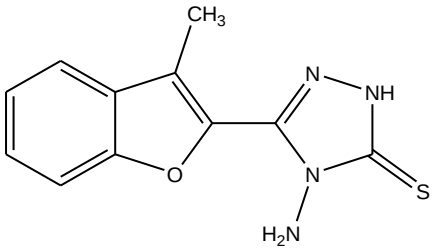
(4)

Ulusoy et al., synthesized 4-(alkylidene/arylidene)amino-2,4- dihydro-5-(2-thienyl)-3H-1,2,4-triazole-3-thiones and explored *in vitro* antibacterial activity against *S. aureus* and *Staphylococcus epidermidis* (Ulusoy et al., 2001). Among the tested compounds, compound with 2-(5-nitro-2-furyl)ethenyl moiety (5) showed antibacterial activity.



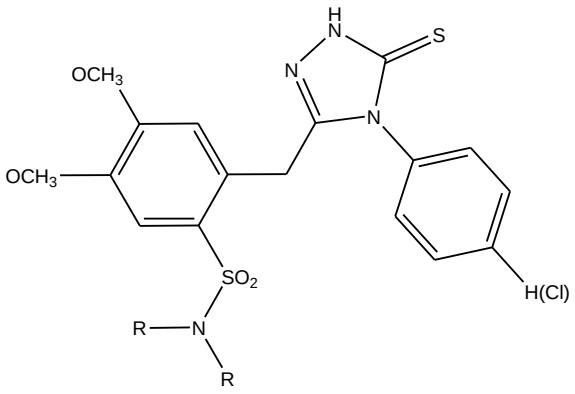
(5)

Dawood et al., synthesized 4-amino-3-(3-methylbenzofuran-2-yl)-1,2,4-triazole- 5-thione (6) and the compound showed antibacterial activity against both *Bacillus cereus* and *Fusarium oxysporium* microorganisms (Dawood et al., 2005).



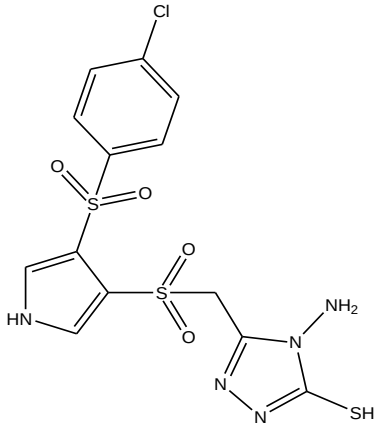
(6)

Ezabadi et al., synthesized 5-[2-(Substitutedsulfamoyl)-4,5-dimethoxy-benzyl]-4-aryl-striazole-3-thiones (7) and evaluated as antibacterial agents (Ezabadi et al., 2008). The derivatives showed antibacterial activity at 100 µg/ml against *E. coli*, the same minimum inhibitory concentration (MIC) as antibiotic streptomycin, but much better than chloramphenicol (MIC at 250.0 µg/ml).



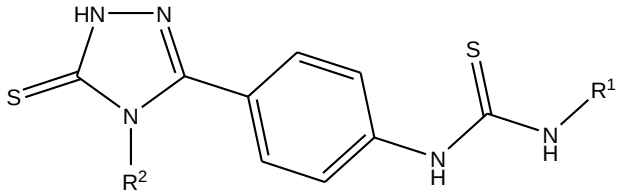
(7)

4'-Amino-5'-(4-arylsulfonyl-1H-pyrrole-3-sulfonylmethyl)-[1',2',4']triazole-3'-thiol were synthesized and tested for their in vitro antimicrobial activity against the Gram-positive bacteria (*S. aureus, B. subtilis*) and the Gram-negative bacteria (*E. coli, K. pneumoniae*) by agar disc-diffusion method using nutrient agar medium. The results of primary screening demonstrated that the inhibitory activity against the gram-positive bacteria was generally higher than that of the gram-negative bacteria. Compound (8) with 4-chlorophenyl substituent was found to have maximum activity (Padmavathi et al 2009).



(8)

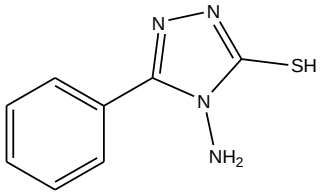
Kucukguzel et al., synthesized a novel derivatives of N-alkyl/aryl-N'-[4-(4-alkyl/aryl-2,4-dihydro-3H-1,2,4-triazole-3- thione-5-yl)phenyl]thioureas (9) (R^1: CH_3 , $CH_2CH=CH_2$, C_6H_{11}; R^2: C_6H_{11}, $CH_2CH=CH_2$, C_6H_5) and evaluated for their antimicrobial activity against *M. tuberculosis* H37Rv as well as *Mycobacterium fortuitum* which was a rapid growing opportunistic pathogen (Küçükgüzel et al., 2001). Among them four synthesized compounds showed same MIC like tobramycin at 32 µg/ml.



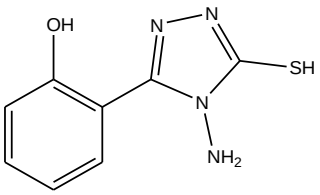
(9)

R1	**R2**
CH_3	C_6H_{11}
$CH_2CH=CH_2$	$CH_2CH=CH_2$
$CH_2CH=CH_2$	C_6H_5
C_6H_{11}	$CH_2CH=CH_2$

Sahoo et al., synthesized a series of 4-amino-5-phenyl-4H- [1,2,4]-triazole-3-thiol derivatives and examined for their bacterial activity against *B. subtilis*, *E. coli*, *P. aeruginosa* and *P. fluorescens* by paper disc diffusion method (Sahoo et al., 2010). From this study it was established that the compounds with free NH_2 groups at the 4-position showed moderate activity. These two compounds (10, 11) were found to have moderate activity at 12.5 µg/ml against tested strain of bacteria as compared with ceftriaxone (5 µg/ml).

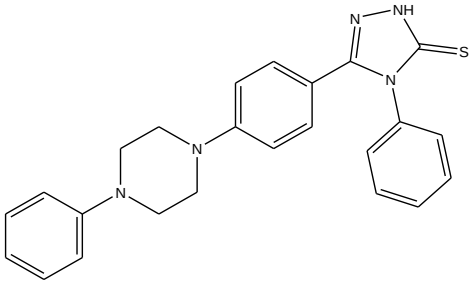


(10)



(11)

Rajaka et al., designed and synthesized new derivatives of [5-(4- substituted-piperazin-1-yl) phenyl]-4-substituted-2,4-dihydro- 1,2,4-triazole-3-thione and tested their antimicrobial activity against different bacteria *P. aeruginosa*, *B. subtilis*, *P. mirabilis* and *S. aureus*. Compound [5-(4-phenyl-piperazin-1-yl)-phenyl]-4-phenyl-2,4-dihydro-1,2,4-triazole-3-thione (12) showed promising antimicrobial activity among all the tested compounds (Rajak et al., 2011).

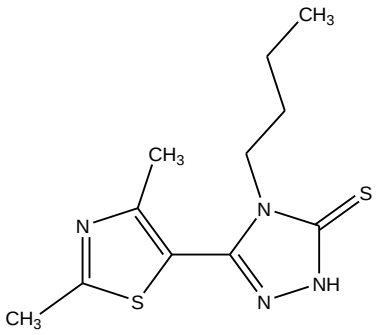


(12)

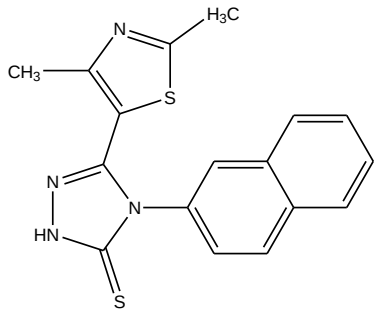
2.2. Antifungal activity

Jalilian et al., synthesized 1,2,3-thiadiazolo-1,2,4-triazoles bearing alkyl or aryl substituent on the triazole ring and determined their antifungal effects. Compounds, with butyl (13) and 2-

naphthyl (14) substituent showed potent activity against *Cryptococcus neoformans* and *Sacchromyces cerevisiae* at MIC ranges of 3.12-6.25 µg/ml but their activities were moderate against *Candida albicans* and weak against *Aspergillus fumigate* (Jalilian et al., 2000).

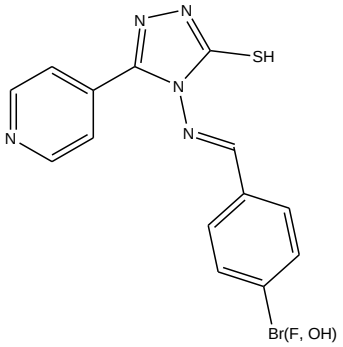


(13)

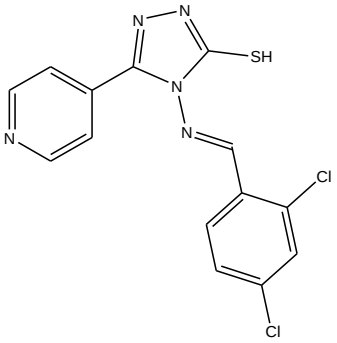


(14)

Sun et al., synthesized schiff bases 4-amino-5-(4-pyridyl)-2,4-dihydro-1,2,4-triazole-3-thione derivatives and evaluated them for their antifungal activity and triadimefon was used as the reference standard (Sun et al., 2007). Among them compounds 15 and 16 exhibited good and broad fungus-inhibiting activities to several vegetable pathogens.



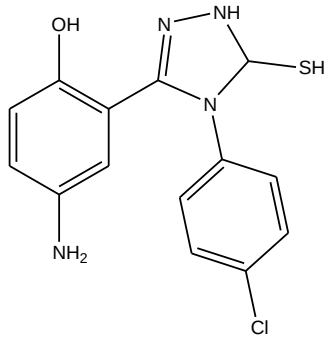
(15)



(16)

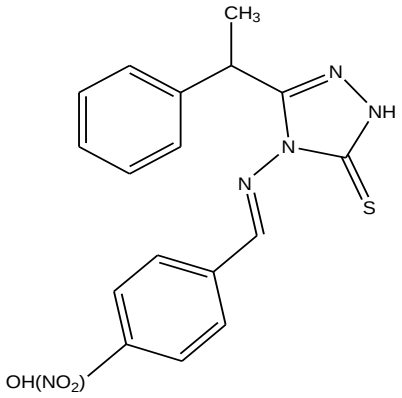
Hussain et al., synthesized 4-amino-2-[4-(4-substitutedphenyl)-5-sulfanyl-4H-1,2,4-triazol-3-yl]phenol and evaluated for their antifungal activity against *A. niger* and ketoconazole was treated as a standard (Hussain et al., 2008). The result showed that 1,2,4-triazole derivatives

having chloro group at *para* position of phenyl ring (17) displayed a MIC of 25 µg/ml against *A. niger*.



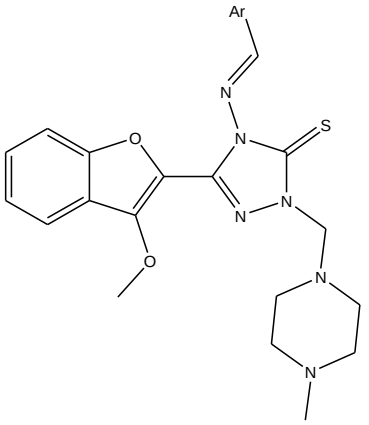
(17)

Çalisir et al., synthesized 4-[[(4-aryl)methylidene]amino]-5-(1-phenylethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione and explored in vitro antifungal activity against *C. albicans*. From the study it was observed that compound containing 4-hydroxy and 4-nitrosubstituted phenyl ring (18) showed good antifungal activity at MIC value of 9 µg/ml (Calisir et al., 2010).



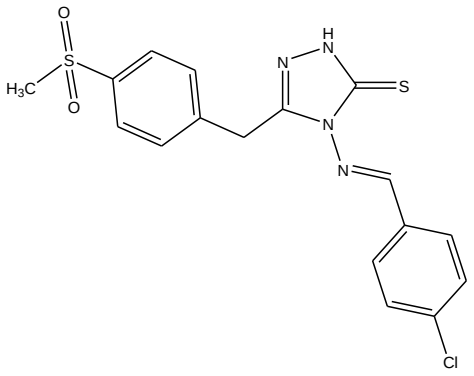
(18)

Ramakrishna et al., synthesized a series of novel Mannich bases derived from 1,2,4-triazoles and evaluated for their antifungal activity and ketoconazole was taken as a reference standard. Compounds with N-methyl piperazine derivatives (19) were showed antifungal activity against *C. albicans* (Ramakrishna et al., 2012).



(19)

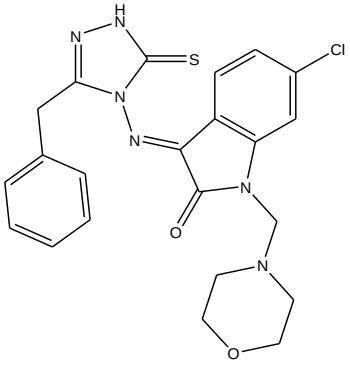
Sumangala et al., reported a new series of 1,2,4-triazole derivatives which were synthesized from 4-amino-5-[4-(methylsulfonyl)benzyl]-2,4-dihydro-3H-[1,2,4]-triazole-3-thione and tested for antifungal activity against *C. albicans.* The results revealed that 4-{[(4-chlorophenyl)methylene]amino}-5-[4-(methylsulfonyl)benzyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione (20) exhibited good anti-fungal activity with MIC=1.562 µg/ml compared to others due to the 4-chlorophenyl substituent (Sumangala et al., 2013).

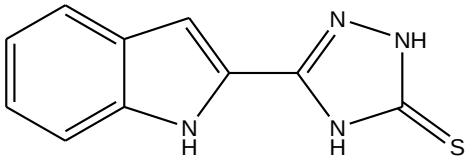


(20)

A series of Schiff and Mannich bases of isatin containing 1,2,4-triazole derivatives were ssynthesized by Murthy et al. and evaluated for antifungal activity. It was found that 3-[(3-benzyl-5-thioxo-1,5-dihydro-(1,2,4)triazol-4-ylimino]-5-chloro-1-morpholin-4-yl-methyl-1,3

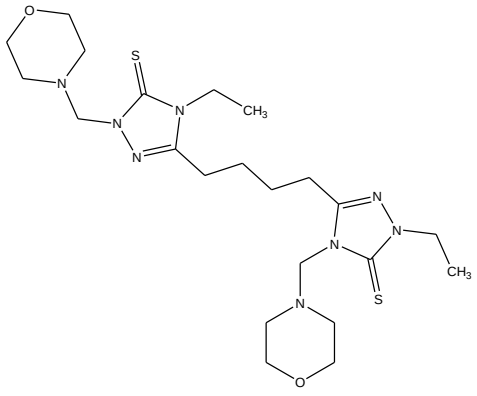
dihydro-indol-2-one (21) showed equal activity against *A. niger* compare to fluconazole. 1,2-Dihydro-5-(1H-indol-2-yl)-1,2,4-triazole-3-thione was synthesized and screened for antifungal activity against *A. niger*, *Fusarium oxysporum* and *Rhizoctonia solani*. This triazole derivative (22) exhibited high inhibition activity at MIC 5 µg/ml against most tested microorganisms (El Sayed et al., 2013).





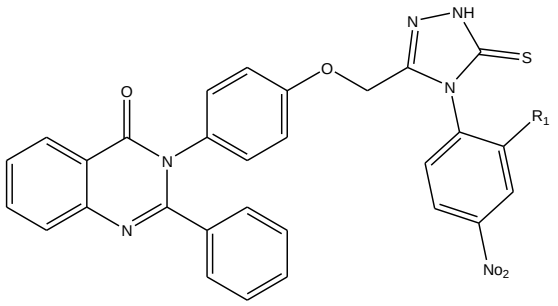
(21) **(22)**

Koparır et al synthesized a new Mannich bases of 5,5'-butane-1,4-diylbis[4-ethyl-2,4-dihydro-3H-1,2,4-triazole-3-thiones and screened against *A. flavus* and *C. albicans*. Fluconazole was used as a standard (Koparir et al., 2013). This study revealed that 5, 5'-butane-1,4-diyl bis[4-ethyl-2-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione] (23) showed excellent antifungal activity at 1.56-3.12 µg/ml against all the tested fungal strains.



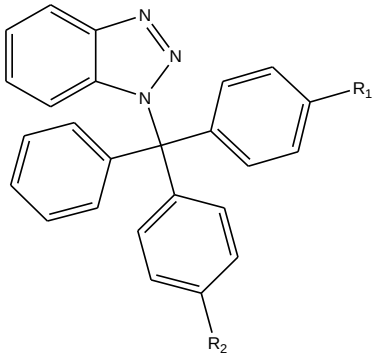
(23)

Freddy *et al.*, synthesized a series of 3-[4-(substituted phenyl-5-thioxo-4, 5-dihydro-1*H*-1, 2, 4-triazole-3-yl-methoxy)-phenyl]-2-phenyl-3*H*-quinazoline-4-ones which were screened for antifungal activity. The compound 3-{4-[-nitrophenyl)-5-thioxo-4, 5 dihydro-1*H*- [1,2,4]triazole-3-yl-methoxy]phenyl}-2-phenyl-3*H*-quinaolin-4-one (24) exhibited good activity against *Aspergillus niger* **(**Havaldar et al., 2008**)**.



(24)

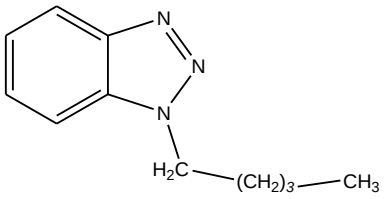
Rezaei *et al.*, described the synthesis and evaluation of novel series of benzotriazoles 25 and 26 as inhibitors of cytochrome P450 14α-demethylase (14DM). Compounds were designed by generating a virtual library and docking them into the enzyme active site. The analogs 25a, 25b, and 25c had low antifungal activity. The activity was decreased by the presence of a methoxy group substituted on the benzotriazole moiety (compounds 25b and 25c). Compound 26a possessed potent inhibitory effect on tested fungi.



(a) $R_1 = R_2 = H$

(b) $R_1 =$ H, $R_2 = OCH_3$

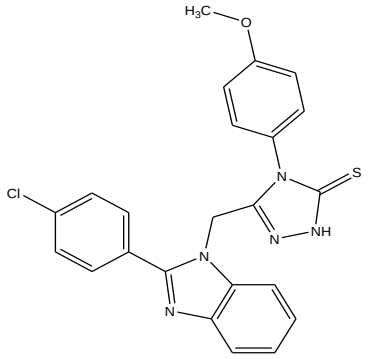
(c) $R_1 = R_2 = OCH_3$

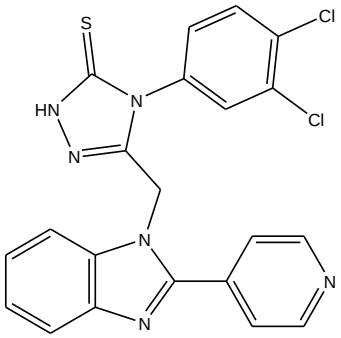


(25) **(26)**

2.3. Antioxidant activity

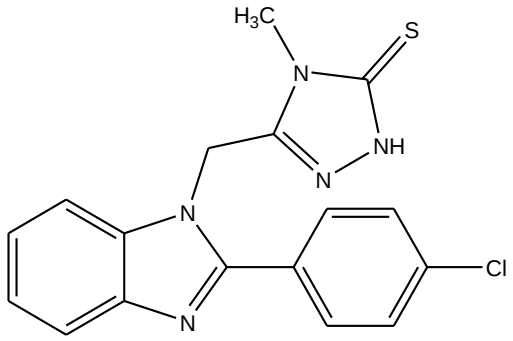
Ayhan-Kilcigil et al., have been synthesized some novel benzimidazole derivatives, having 1,2,4- triazole-3-thiones and evaluated for their antioxidant effect (Ayhan-KIlcigİl et al., 2005) by DPPH method. Among them 5-(2-(p-Chlorophenyl)benzimidazol-1-ylmethyl)-4-methoxyphenyl-2,4-dihydro-1,2,4-triazole-3-thione (27) and 5-(2-(4-pyridinyl)benzimidazol-1-ylmethyl)-3,4- dichlorophenyl-2,4-dihydro-1,2,4-triazole-3-thione (28) showed highest activity with IC_{50} values of 5.4×10^5 , 1.1×10^4, respectively. The IC_{50} values for these compounds were smaller than that for butylhydroxytoluene.





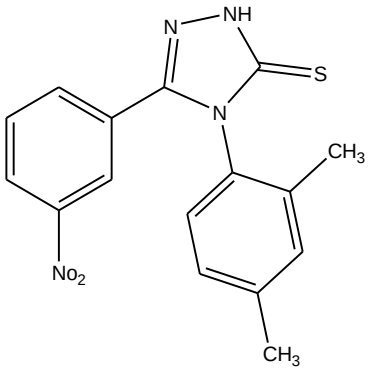
(27) **(28)**

Kus et al., have synthesized a series of 5-[(2-(substitutedphenyl)-1H-benzimidazol-1-yl) methyl-4-methyl-1,2,4-triazole-3(4H)-thiones and explored their scavenger effects on DPPH radical, superoxide radical, and also examined the inhibition capacity of their microsomal ethoxyresorufin O-deethylase (EROD) activity (Kuş et al., 2008). All triazole compounds had moderate effect on DPPH radical with IC_{50} values ranging from 140 to 605 mM. On the other hand Compound, with 4-Cl substituent (29) inhibited the EROD activity with an $IC_{50} = 4.5 \times 10^{4}$ M, which is similarly better than the specific inhibitor caffeine $IC_{50} = 5.2 \times 10^{-4}$ M.

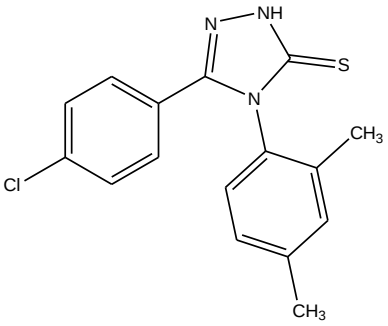


(29)

Khan et al have been reported a series of novel 4,5-disubstituted-2,4-dihydro-3H-1,2,4-triazole-3-thiones and examined for their antioxidant activity by DPPH method (Khan et al., 2010). Compounds 4-(2,4-Dimethylphenyl)-5-(3-nitrophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (30) and 5-(4-chlorophenyl)-4-(2,4-dimethylphenyl)-2,4-dihydro-3H-1,2,4- triazole-3-thione (31) showed better activity with the IC_{50} values 242.49 ± 3.64 and 292.38 ± 1.60 mM respectively.



(30)

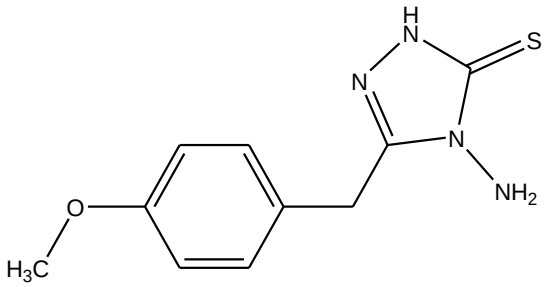


(31)

A group of 4-amino-5-aryl-3H-1,2,4-triazole-3-thiones, bearing various methoxybenzyl- and methoxyphenethyl were synthesized by Hanif et al and evaluated for their antioxidant activity using DPPH radical. Among them compound 4-Amino-3-(4-methoxybenzyl)-1,2,4-triazole-5(4H)-thione (32) showed hydrogen donating ability on reaction with DPPH radical with the IC_{50} value of 51.8 mM (Hanif et al., 2012).

Aswathanarayanappa et al. have been synthesized schiff based 1,2,4- triazole and screened for antioxidant properties by free radical scavenging, lipid peroxidation, anti-hemolytic activity, and their protective effects against DNA oxidative damage (Aswathanarayanappa et al., 2013). Compound 2-(-{[3-(4-Chloro-2-methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl] imino}methyl)benzene-1,4-diol (33) showed highest effect against H_2O_2-induced hemolysis of human erythrocytes. Compound 5-(4-Chloro-2-methylphenyl)-4-[(4-methoxybenzylidene)amino]-4H1,2,4-triazole-3-thiol (34) was found to be effective to inhibit lipid peroxidation. Compound 4-[{[3-(4-chloro-2- methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl]imino}methyl] phenol (35) inhibit DNA oxidative damage. Among them two derivatives (33, 35) were highly potent antioxidant agents. They showed promising DPPH radical scavenging activity in the level of inhibition 89.2% and 86.8%, respectively.

Nadeem et al had been synthesized a novel 4,5-disubstituted-1,2,4-triazole-3-thiones derivatives and evaluated for their antioxidant activity (Nadeem et al., 2013). Compounds 4-Hexyl-2,4-dihydro-5-(3-pyridyl)-3H-1,2,4-triazole-3-thione, (36) (92.5%) and 5-benzyl-4-hexyl-2,4-dihydro-3H-1,2,4- triazole-3-thione, (37) (92.3%) showed significant decrease in the concentration of DPPH radical due to the scavenging ability of these compounds.



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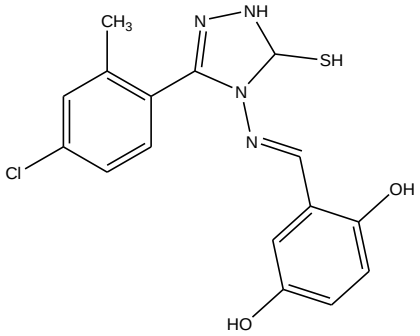

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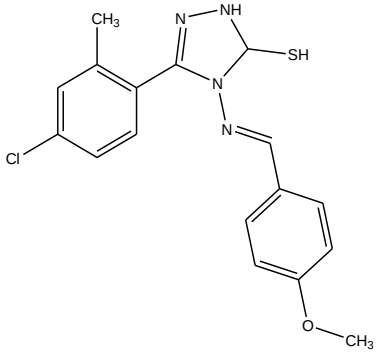

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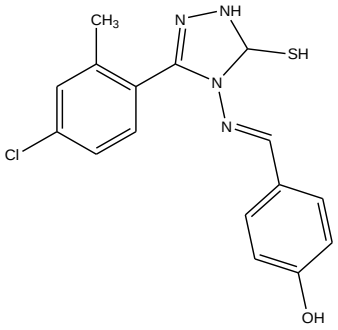

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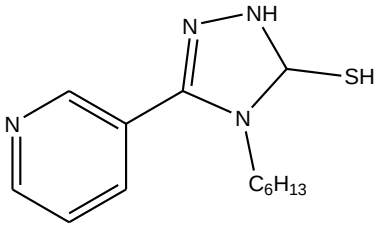

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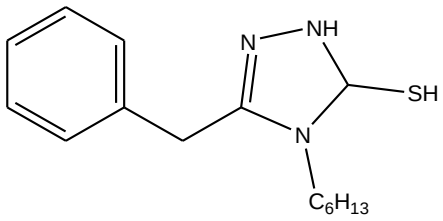
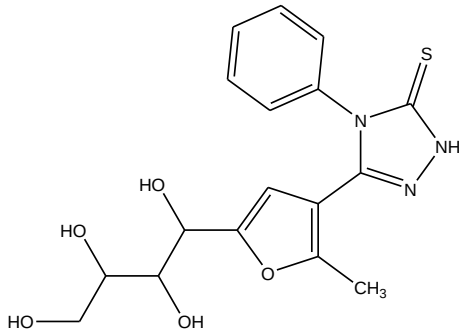


(37)

El Sadek et al have been synthesized new aromatic C-nucleoside derivatives with triazole moiety and screened for their antioxidant activity by DPPH assay, vitamin E was used as standard (EC_{50} 0.705) (El Sadek et al., 2014). Compounds 5-(5-(10,20,30,40-tetrahydroxybutyl)-2-methylfuran-3-yl)-4-phenyl-2H-1,2,4-triazole-3(4H)-thione, (38) and 4-(4,5-dihydro-4-phenyl-5-thioxo-1H-1,2,4-triazol-3-yl)-5-methylfuran-2-carbaldehyde, (39) were exhibited nearly the same activities (EC_{50} 0.720) and (EC_{50} 0.725) respectively compared with the standard.



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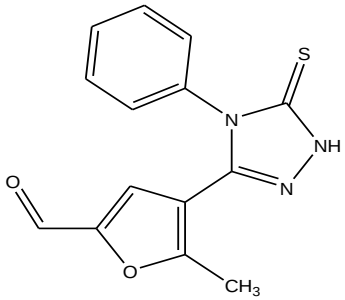
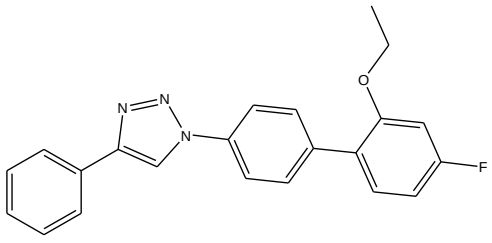


(39)

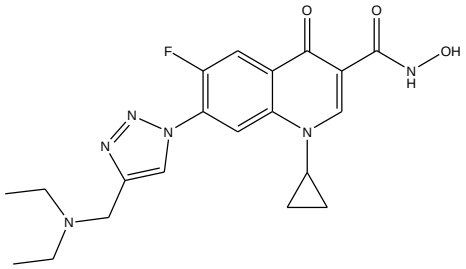
2.4. Anticancer activity

A series of 1,4-disubstituted 1,2,3-triazoles and 1,2,4-triazolo[1,5-a]pyrimidine derivatives via copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction have been synthesized by Gilandoust et al and evaluated for their anticancer activity against MCF7 cells. Compound 1-(2′-ethoxy-4′-fluoro-[1,1′-biphenyl]-4-yl)-4-phenyl-1H-1,2,3-triazole (45) showed cytotoxic against MCF7 cell lines (IC_{50} value of 1.69 mM) (Gilandoust et al., 2018).

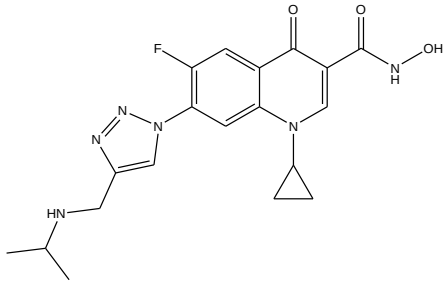


(45)

Rajulu et al synthesized some hydroxamic acid derivatives at C-3 position of fluoroquinolones and screened for their *in vitro* antimicrobial and anti-proliferative activity. Out of these, Compounds 46 and 47 showed potent activity against A549 (Lung adenocarcinoma) and HCT-116 (Colon carcinoma) cell lines (Govinda Rajulu et al., 2014).

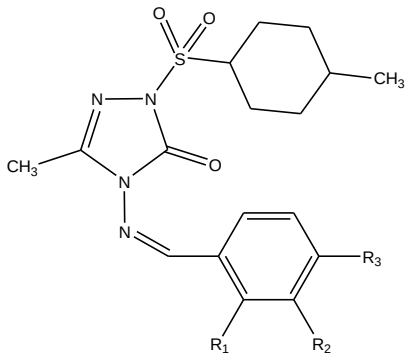


(46)



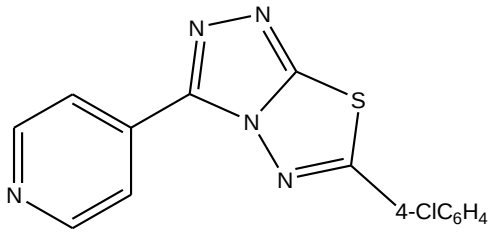
(47)

Ikram Saadaoui et al have been synthesized a series of 4-amino-1,2,4-triazole derivatives (48). All the compounds showed excellent activity with a range of 71.22 ± 0.98, 96.42 ± 0.02% at 1.20 mM (Saadaoui et al., 2019).



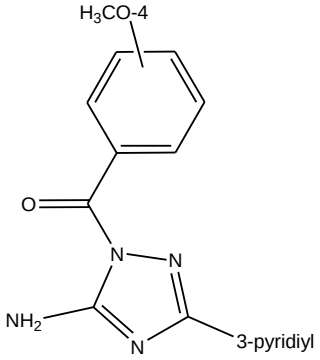
(48)

A series of new N-substituted-3-mercapto-1,2,4-triazoles, triazolo [1,3,4] thiadiazines and triazolo[1,3,4]thiadiazoles have been synthesized by Kamel et al. Among of them compound 49 have alike cytotoxic effect to the standard CHS 828 against on gastric cancer cell line (IC_{50} = 25 nM) (Kamel et al., 2014).



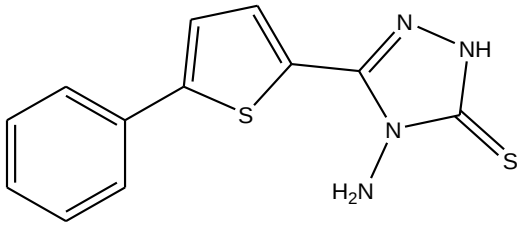
(49)

A series of novel 1,2,4-triazole scaffold were reported by El-Sherief et al. and screened for their anticancer activity using MTT assay. Compound 50 showed the most excellent EGFR inhibition with IC_{50} = 3.6 mM (El-Sherief et al., 2018).



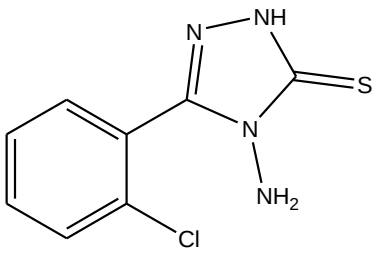
(50)

Mavrova et al., synthesized a series of 4,5-substituted-1,2,4-triazole-thiones and screened *in vitro* cytotoxic effect against thymocytes. Compounds 4-Amino-5-(5-phenylthiene-2-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (51) showed significant cytotoxic activity with IC_{50} = 0.012 mM (Mavrova et al., 2009).



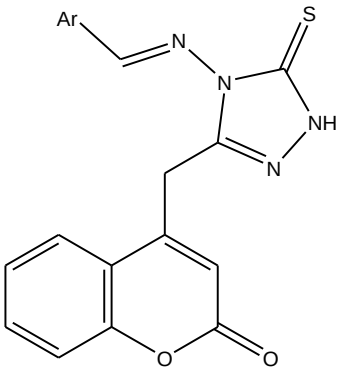
(51)

Singha et al., have been synthesized 4-amino-5-mercapto-3-(2-chlorophenyl)-1,2,4-triazole (52) and examined for anticancer activity. The compound showed promising anticancer activity on Ehrlich Ascites Carcinoma (EAC) bearing mice where 5- fluorouracil was used as standard (Singha et al., 2012).

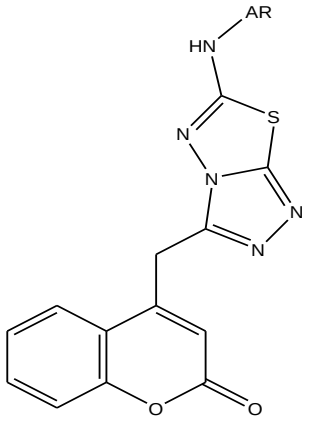


(52)

Al-Wahaibi et al., have been reported a series of novel coumarin derivatives carrying 1,2,4-triazole or 1,2,4-triazolo thiadiazole moieties and explored *in vitro* for anticancer activity against human colon cancer (HCT116) cell line. Compounds 53 and 54 exhibited noticeable anticancer activity with IC_{50} values 4.363 and 2.656 μM, respectively (Al-Wahaibi et al., 2018).



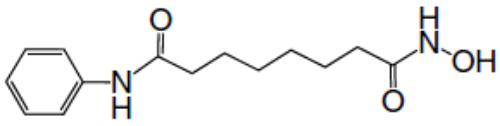
(53) Ar= 4-OH, 3-$MeOC_6H_3$



(54) Ar= 4-$H_2NSO_2C_6H_4$

According to PA Marks, suberoylanilide hydroxamic acid (SAHA) can cause growth arrest and death of a broad variety of transformed cells both *in vitro* and *in vivo* at concentrations that have

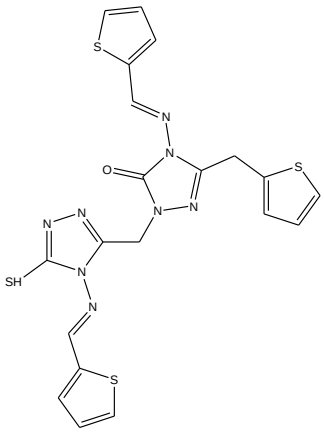
little or no toxic effects on normal cells. In the new promising class of anticancer drugs SAHA (vorinostat) is the lead compound. SAHA is in clinical trials and has significant anticancer activity against both hematologic and solid tumors at doses well tolerated by patients. It has been already approved that SAHA is used for the treatment of cutaneous T-cell lymphoma (Marks et al., 2007).



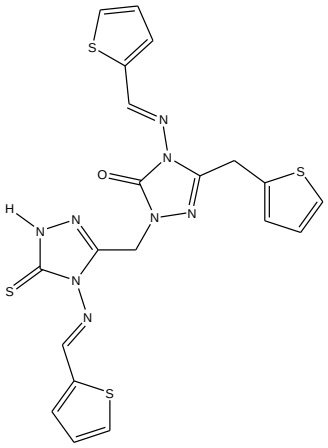
SAHA

2.5. Antileishmanial activity

A series of thiol-thione tautomerism of 1,2,4-triazole derivative with Schiff base have been reported by Suleymanoglu et al. and explored for their *in vitro* antileishmanial activity against *Leishmania infantum* promastigots. The results showed that 1,2,4-triazole derivative exists in both thiol and thione form (55, 56) and, can be used as antiparasitic agent (Süleymanoğlu et al., 2017).

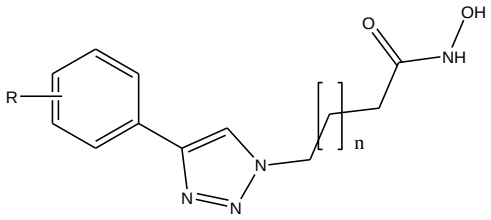


(55)

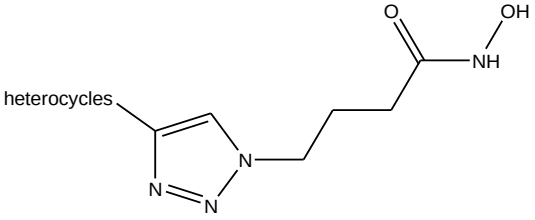


(56)

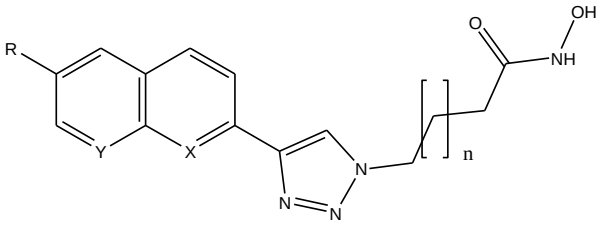
The triazole-hydroxamic acid hybrids (57-59) showed promising in vitro antileishmanial and the IC_{50} values of most of hybrids were in ng/ml level.



(57)



(58)



(59)

Chapter 3

3. Objectives.

Triazole is getting more important nowadays due to its high efficacy and better safety profiles. Several synthetic routes have been discovered for the synthesis of triazole and they are economically advantageous with their low production cost. Triazoles have a broader spectrum of applications compared to imidazoles leading to this class of azoles being. 1,2,4-Triazole derivatives have different biological activities, like as antifungals (Itraconazole, Fluconazole), anticancer, in migraine headaches (Rizatriptan), anti-inflammatory, antihypertensive etc. Due to their wide range of biological, agrochemical and chemical properties, they have drawn the attention to the scientists.

In the area of coordination chemistry, hydroxamic acids are excellent ligands. They deprotonate to give hydroxamates, which bind to metals ions as bidentate ligands. So high is the affinity of hydroxamates for ferric ions that nature has evolved families of hydroxamic acids to function as iron-binding compounds (siderophores) in bacteria. Hydroxamic acids are successful molecules in the field of cancer chemotherapy. Suberoylanilide Hydroxamic Acid (SAHA), Belinostat & panobinostat are the hydroxamates based drugs for cancer chemotherapy, approved by the U.S. Food and Drug Administration (FDA. Some others are also in clinical trials, like m-carboxycinnamic acid bishydroxamic acid, SB-939 (phase II) and 4SC-201. SAHA (Suberoylanilide Hydroxamic Acid) is considered as a potent anticancer agent among all hydroxamic acid derivatives because of their very good chelating capability, That's why the hydroxamates group grabs the attraction for scientists from all over the world.

Based upon the fact the 1,2,4-triazole linked hydroxamic acid derivatives were synthesized for the treatment of different diseases involving the following objectives:

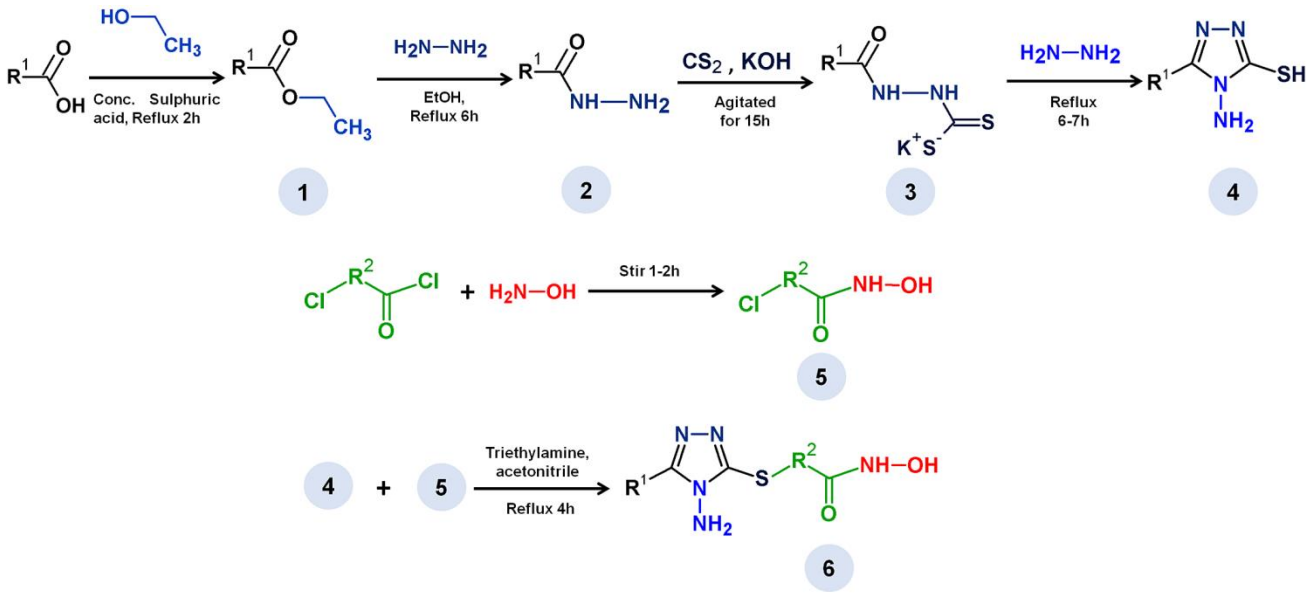
- Synthesis of a few 1,2,4-triazole-linked hydroxamic acid derivatives.

- Characterization of the synthesized compounds by means of elemental and various spectrophotometric analysis.
- Evaluation of *in vitro* antibacterial activity.
- Evaluation of *in vitro* antifungal effect.
- Evaluation of *in vitro* antioxidant activity.
- Evaluation of *in vivo* and *in vitro* anticancer activity.
- Evaluation of *in vitro* antileishmanial activity.

Chapter 4

4. Synthetic procedure

A series of novel 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide and 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide(6a-6l) compounds were synthesized by reacting 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol with the respective 2-chloro-N-hydroxy acetamides/ 3-chloro-N-hydroxypropanamide (Fig 7). Briefly, the reaction was carried out in three parts i.e. synthesis of 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol and synthesis of 2-chloro-N-hydroxy acetamides/ 3-chloro-N-hydroxypropanamide then both were coupled to synthesis of the final compounds. 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol were synthesized by Reid-Heindal method (Reid et al., 1976) i.e. aryl acids were taken as a starting material and refluxed for 2 h with ethanol and concentrated sulfuric acid to produce the corresponding aryl esters. The aryl esters were further refluxed with hydrazine hydrate to yield corresponding aryl hydrazides. Then in presence of ethanolic potassium hydroxide, the aryl hydrazides were condensed with carbon-di-sulfide to produce potassium-3-aroyldithiocarbazates. These aroyldithiocarbazates were cyclized to produce corresponding aryl substituted 1,2,4-triazole. In another part, chloroacetyl chloride or chloropropionyl chloride reacted with hydroxyl amine hydrochloride to yield 2-chloro-N-hydroxy acetamides or 3-chloro-N-hydroxypropanamide, respectively. In the final step, 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxy acetamides or 3-chloro-N-hydroxypropanamide were condensed in presence of triethylamine to produce the final compound i.e. 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide or 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide; respectively. All the final compounds (6a-6l) were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and mass spectral data. The synthetic route of the proposed scaffold was depicted in figure 7.



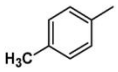
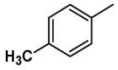
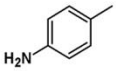
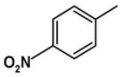
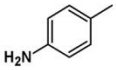
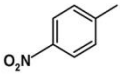
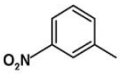
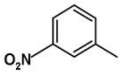
Compound code	R^1	R^2	Compound code	R^1	R^2
6a		$-CH_2-$	6g		$-CH_2-$
6b		$-CH_2-CH_2-$	6h		$-CH_2-CH_2-$
6c		$-CH_2-$	6i		$-CH_2-$
6d		$-CH_2-CH_2-$	6j		$-CH_2-CH_2-$
6e		$-CH_2-$	6k		$-CH_2-$
6f		$-CH_2-CH_2-$	6l		$-CH_2-CH_2-$

Fig 7. Schematic representation of synthetic procedure

Ethyl pyridine-3-carboxylate (1a)

Nicotinic acid (0.16 mol) was taken in a 250 ml round bottom flask (rbf). 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with small amount of

activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: 15.5 g (77.5%)

Ethyl-4-aminobenzoate (1b)

4-Amino benzoic acid (0.145 mol) was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with small amount of activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: 17.9 g (64.5 %)

Ethyl-2-sulfanylbenzoate (1c)

2-Sulfanyl benzoic acid (0.129 mol) was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with small amount of activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: 15.2 g (76 %)

Ethyl-4-methylbenzoate (1d)

4-Methyl benzoic acid (0.15 mol) was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with small amount of activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: 17 g (81%)

Ethyl-4-nitrobenzoate (1e)

4-Nitro benzoic acid (0.09 mol) was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with small amount of activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: 10 g (66.67 %)

Ethyl-3-nitrobenzoate (1f)

3-Nitro benzoic acid (0.12 mol) was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. Sulphuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The

combined ether fraction was collected and kept overnight with small amount of activated anhydrous sodium sulphate. The ester in the form of oil was collected by means of distilling out the ether.

Yield: (16.7g) 83.5 %

Pyridine-3-carbohydrazide (2a)

Ethyl pyridine-3-carboxylate (0.079 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.079 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 9.2 g (76.67%)

Analysis: FTIR (KBr) (cm^{-1}): 3425.79 (N-H stretching), 3097.69 (aromatic C-H stretching), 1672.53 (C=O stretching).

4-aminobenzohydrazide (2b)

Ethyl-4-amino benzoate (0.06 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.06 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 7.1 g (71%)

Analysis: FTIR (KBr) (cm^{-1}): 3419.56 (N-H stretching), 3027.27 (aromatic C-H stretching), 1678.88 (C=O stretching).

2-sulfanylbenzohydrazide (2c)

Ethyl-2-Sulfanylbenzoate (0.066 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.066 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 8.7 g (72.5%)

Analysis: FTIR (KBr) (cm^{-1}): 3392.75 (N-H stretching), 3064.16 (aromatic C-H stretching), 1694.69 (C=O stretching).

4-methylbenzohydrazide (2d)

Ethyl-4-methylbenzoate (0.091 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.091 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 12 g (80%)

Analysis: IR (KBr) (cm-1): 3386.68 (N-H stretching), 3082.63 (aromatic C-H stretching), 1643.74 (C=O stretching).

4-nitrobenzohydrazide (2e)

Ethyl-4-nitrobenzoate (0.041 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.041 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room

temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 5.3 g (66.25%)

Analysis: FTIR (KBr) (cm^{-1}): 3323.76 (N-H stretching), 3074.19 (aromatic C-H stretching), 1708.60 (C=O stretching).

3-nitrobenzohydrazide(2f)

Ethyl-3-nitrobenzoate (0.076 mol) was dissolved in 30 ml of ethanol and hydrazine hydrate (0.076 mol) was added drop wise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried and recrystallized from dehydrated ethanol to yield white crystals.

Yield: 11.8 g (78.66%)

Analysis: FTIR (KBr) (cm^{-1}): 3430.23 (N-H stretching), 3097.39 (aromatic C-H stretching), 1672.89 (C=O stretching).

Potassium 2-nicotinoylhydrazine-1-carbodithioate (3a)

Potassium hydroxide (0.051mol) was dissolved in 75 ml of absolute alcohol and Pyridine-3-carbohydrazide (0.051mol) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulphide was added (0.051mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 3.8 g (54.28%)

Analysis: FTIR (KBr) (cm^{-1}): 3403.31 (N-H stretching), 3023.76 (aromatic C-H stretching), 1691.54 (C=O stretching), 1536.47 (C=N stretching), 1135.49 (C=S stretching).

Potassium 2-(4-aminobenzoyl) hydrazine-1-carbodithioate (3b)

Potassium hydroxide (0.0397 mol) was dissolved in 75 ml of absolute alcohol and 4-Aminobenzohydrazide (0.0397 mol) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulphide was added (0.0397 mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 3.7 g (61.67%)

Analysis: FTIR (KBr) (cm^{-1}): 3346.92 (N-H stretching), 3032.35 (aromatic C-H stretching), 1687.57 (C=O stretching), 1513.80 (C=N stretching), 1171.33 (C=S stretching).

Potassium 2-(2-mercaptobenzoyl)hydrazine-1-carbodithioate (3c)

Potassium hydroxide (0.0357mol) was dissolved in 75 ml of absolute alcohol and 2-mercaptobenzohydrazide (0.0357mol) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulphide was added (0.0357mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 4.2 g (70%)

Analysis: FTIR (KBr) (cm^{-1}): 3382.54 (N-H stretching), 3090.87 (aromatic C-H stretching), 1614.34 (C=O stretching), 1497.14 (C=N stretching), 1190.35 (C=S stretching).

Potassium 2-(2-methylbenzoyl)hydrazine-1-carbodithioate (3d)

Potassium hydroxide (0.066 mol) was dissolved in 75 ml of absolute alcohol and 2-methylbenzohydrazide (0.066 mol) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulphide was added (0.066 mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 5.8 g (58%)

Analysis: FTIR (KBr) (cm^{-1}): 3411.26 (N-H stretching), 3069.59(aromatic C-H stretching), 1697.88 (C=O stretching), 1511.99 (C=N stretching), 1180.05 (C=S stretching).

Potassium 2-(4-nitrobenzoyl)hydrazine-1-carbodithioate (3e)

Potassium hydroxide (0.0276mol) was dissolved in 75 ml of absolute alcohol and 4-nitrobenzohydrazide (0.0276 mol) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulphide was added (0.0276 mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 2.7 g (54%)

Analysis: FTIR (KBr) (cm^{-1}): 3479.52 (N-H stretching), 3081.67 (aromatic C-H stretching), 1721.62 (C=O stretching), 1572.90 (C-N stretching), 1344.47 (C=S stretching).

Potassium 2-(3-nitrobenzoyl)hydrazine-1-carbodithioate (3f)

Potassium hydroxide (0.055mol) was dissolved in 75 ml of absolute alcohol and 3-nitrobenzohydrazide (0.055mol) was added to the above solution. The mixture was then cooled

in an ice-bath. To this mixture carbon disulphide was added (0.055mol) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

Yield: 6.4 g (64%)

Analysis: FTIR (KBr) (cm^{-1}): 3317.81 (N-H stretching), 3076.51 (aromatic C-H stretching), 1692.95 (C=O stretching), 1510.64 (C=N stretching), 1294.51 (C=S stretching).

4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazole-3-thiol (4a)

A suspension of Potassium 2-nicotinoylhydrazine-1-carbodithioate (0.012 mol) in 20 ml of water and hydrazine hydrate (0.0358 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 1.8 g (60%)

Analysis: FTIR (KBr) (cm^{-1}): 3342.62 (N-H stretching), 3046.02 (aromatic C-H stretching), 1684.35 (C=O stretching), 1530.63 (C=N stretching), 1431.06 (aromatic C=C stretching), 1303.78 (N-N=C)

4-amino-5-(4-aminophenyl)-4H-1,2,4-triazole-3-thiol (4b)

A suspension of Potassium 2-(4-aminobenzoyl)hydrazine-1-carbodithioate (0.0094 mol) in 20 ml of water and hydrazine hydrate (0.0283 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly

acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 1.6 g (64%)

Analysis: FTIR (KBr) (cm^{-1}): 3420.05 (N-H stretching), 3037.56 (aromatic C-H stretching), 1678.95 (C=O stretching) 1513.17 (C=N stretching), 1441.10 (aromatic C=C stretching), 1309.74 (N-N=C)

4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazole-3-thiol (4c)

A suspension of Potassium 2-(2-mercaptobenzoyl) hydrazine-1-carbodithioate (0.0156 mol) in 20 ml of water and hydrazine hydrate (0.0468 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 2 g (66.7%)

Analysis: FTIR (KBr) (cm^{-1}): 3383.91 (N-H stretching), 3085.29 (aromatic C-H stretching), 1696.04 (C=O stretching), 1563.06 (C=N stretching), 1435.33 (aromatic C=C stretching), 1286.32 (N-N=C)

4-amino-5-(p-tolyl)-4H-1,2,4-triazole-3-thiol (4d)

A suspension of Potassium 2-(4-methylbenzoyl) hydrazine-1-carbodithioate (0.0189 mol) in 20 ml of water and hydrazine hydrate (0.0567 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly

acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 2.5 g (50%)

Analysis: FTIR (KBr) (cm^{-1}): 3404.15 (N-H stretching), 3074.21 (aromatic C-H stretching), 1697.37 (C=O stretching), 1512.25 (C=N stretching), 1439.92 (aromatic C=C stretching), 1269.93 (N-N=C)

4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazole-3-thiol (4e)

A suspension of Potassium 2-(4-nitrobenzoyl) hydrazine-1-carbodithioate (0.0067 mol) in 20 ml of water and hydrazine hydrate (0.0201 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 1 g (50%)

Analysis: FTIR (KBr) (cm^{-1}): 3430.52 (N-H stretching), 3076.39 (aromatic C-H stretching), 1697.97 (C=O stretching), 1511.29 (C=N stretching), 1413.99 (aromatic C=C stretching), 1269.76 (N-N=C)

4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazole-3-thiol (4f)

A suspension of Potassium 2-(3-nitrobenzoyl)hydrazine-1-carbodithioate (0.017 mol) in 20 ml of water and hydrazine hydrate (0.051 mol) was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added drop wise to make the reaction mixture strongly acidic. The

precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

Yield: 2.3 g (57.5%)

Analysis: FTIR (KBr) (cm^{-1}): 3386.52 (N-H stretching), 3084.74 (aromatic C-H stretching), 1692.85 (C=O stretching), 1511.04 (C=N stretching), 1462.47 (aromatic C=C stretching), 1295.99 (N-N=C)

2-chloro-N-hydroxyacetamide (5a)

0.01 mol of chloro acetyl chloride was dissolved in 30 ml of methanol. To the reaction mixture, hydroxylamine stock solution (0.04 mol of hydroxylamine hydrochloride was dissolved in 12 ml of hot methanol. The mixture was stirred for 5 min and to it, a solution of 0.06 mol potassium hydroxide in 10 ml of methanol was added drop wise. The resulting solution was cooled to room temperature and filtered to obtain 2 mol of stock solution) was added drop wise and the mixture was stirred for 2 h. The precipitate thus obtained, was washed with ether and recrystallized from methanol.

Yield: 2.72 g (70%)

Analysis: FTIR(KBr) (cm^{-1}): 3314.25(N-H stretching), 2999.22 (aliphatic C-H stretching), 1682.05 (C=O stretching), 646.58 (C-Cl stretching).

3-chloro-N-hydroxypropanamide (5b)

0.01 mol of 3-chloro propionylchloride was dissolved in 40 ml of methanol. To the reaction mixture, hydroxylamine stock solution (0.04 mol of hydroxylamine hydrochloride was dissolved in 12 ml of hot methanol. The mixture was stirred for 5 min and to it, a solution of 0.06 mol potassium hydroxide in 10 ml of methanol was added drop wise. The resulting solution was cooled to room temperature and filtered to obtain 2 mol of stock solution) was added drop wise

and the mixture was stirred for 2 h. The precipitate thus obtained, was washed with ether and recrystallised from methanol.

Yield: 2.72 g (70%)

Analysis: FTIR(KBr) (cm^{-1}): 3321.06 (N-H stretching), 3001.14 (aliphatic C-H stretching), 1690.68 (C=O stretching), 646.98 (C-Cl stretching).

2-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6a)

To an equimolar mixture of 4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.2 g (70%), mp: 200-202 °C

Analysis: FTIR (KBR) (cm^{-1}): 3420.39 (N-H stretching), 3028.37 (aromatic C-H stretching), 2981.27 (aliphatic C-H stretching), 1431.09 (aromatic C=C stretching), 1618.65 (C=O stretching), 1530.36 (C=N stretching), 1303.91 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0370 (s; NH), 9.1331 (s; OH), 8.3740-7.5855 (m; Ar- H), 5.7858 (s; NH_2), 1.0373 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4713 (CH), 147.5985 (CH), 124.2174 (CH), 133.6890 (C), 148.3817 (C), 30.6083 (CH2), 167.2465 (C). Mass: [EI+] ($C_9H_{10}N_6O_2S$) 266 Da; Calculated for $C_9H_{10}N_6O_2S$, C, 40.60; H, 3.79; N, 31.56; O, 12.02; S, 12.04 Found: C, 40.72; H, 3.73; N, 31.60; O, 11.96; S, 11.98.

3-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6b)

To an equimolar mixture of 4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1g (73%), mp: 197-200 °C

Analysis: FTIR (KBR) (cm^{-1}): 3412.17 (N-H stretching), 3026.40 (aromatic C-H stretching), 2930.53 (aliphatic C-H stretching), 1431.34 (aromatic C=C stretching), 1618.84 (C=O stretching), 1530.61 (C=N stretching), 1304.29 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0378 (s; NH), 9.1305 (s; OH), 8.3704-7.5807 (m; Ar- H), 5.7834 (s; NH_2), 1.1560-1.0098 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4718 (CH), 147.5995 (CH), 124.2179 (CH), 133.6898 (C), 148.3825 (C), 30.6088 (CH_2), 25.7294 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; Calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 Found: C, 42.92; H, 4.36; N, 29.93; O, 11.39; S, 11.4.

2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6c)

To an equimolar mixture of 4-amino-5-(4-aminophenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid

residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 720 mg (768%), mp: 212-215 °C

Analysis: FTIR (KBR) (cm^{-1}): 3372.34 (N-H stretching), 3038.50 (aromatic C-H stretching), 2918.46 (aliphatic C-H stretching), 1499.91 (aromatic C=C stretching), 1693.64 (C=O stretching), 1536.51 (C=N stretching), 1334.46 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.80011 (s; NH), 9.32056 (s; OH), 7.62590-6.52428 (m; Ar- H), 5.91882 (s; NH_2), 1.24555 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8490 (CH), 120.9804 (CH), 128.4892 (CH), 147.9976 (C), 148.7785 (C), 30.6102 (CH_2), 167.2496 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; Calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 Found: C, 42.53; H, 4.42; N, 29.82; O, 11.26; S, 11.57.

3-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6d)

To an equimolar mixture of 4-amino-5-(4-aminophenyl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 850 mg (71%), mp: 210-214 °C

Analysis: FTIR (KBR) (cm^{-1}): 3341.13 (N-H stretching), 3054.69 (aromatic C-H stretching), 2899.90 (aliphatic C-H stretching), 1442.34 (aromatic C=C stretching), 1678.97 (C=O stretching), 1574.30 (C=N stretching), 1271.78 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.7077 (s; NH), 9.4001 (s; OH), 7.7603-7.2099 (m; Ar- H), 5.2626 (s; NH_2),

1.2561 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8498 (CH), 128.4889 (CH), 120.9809 (C), 147.9981 (C), 148.7789 (C), 30.6109 (CH_2), 25.3169 (CH_2), 167.2498 (C). Mass: [EI+] ($C_{11}H_{14}N_6O_2S$) calc. 294.09 Da, Found: 294 Da; Calculated for $C_{11}H_{14}N_6O_2S$, C, 44.89; H, 4.79; N, 28.55; O, 10.87; S, 10.89 Found: C, 45.05; H, 4.36; N, 28.76; O, 11.09; S, 10.74.

2-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6e)

To an equimolar mixture of 4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.12g (70%), mp: 105-109 °C

Analysis: FTIR (KBR) (cm^{-1}): 3367.57 (N-H stretching), 3082.03 (aromatic C-H stretching), 2980.41 (aliphatic C-H stretching), 1458.46 (aromatic C=C stretching), 1696.7 (C=O stretching), 1563.15 (C=N stretching), 1287.18 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.98857 (s; NH), 9.58518 (s; OH), 8.04105-7.36677 (m; Ar- H), 5.31007 (s; NH_2), 1.36065 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1776 (CH), 126.1872 (CH), 127.2028 (CH), 131.2604 (C), 133.5519 (C), 138.5647 (CH), 148.3655 (C), 30.6009 (CH_2), 165.5395 (C). Mass: [EI+] ($C_{10}H_{11}N_5O_2S_2$) calc. 297.04Da, Found: 297 Da; Calculated for $C_{10}H_{11}N_5O_2S_2$, C, 40.39; H, 3.73; N, 23.55; O, 10.76; S, 21.57 Found: C, 40.45; H, 4.34; N, 29.94; O, 11.21; S, 11.52.

3-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6f)

To an equimolar mixture of 4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed

for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.3 g (74%), mp: 100-103°C

Analysis: FTIR (KBR) (cm^{-1}): 3385.76 (N-H stretching), 3093.54 (aromatic C-H stretching), 2980.07 (aliphatic C-H stretching), 1458.33 (aromatic C=C stretching), 1695.47 (C=O stretching), 1562.97 (C=N stretching), 1286.84 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.00011 (s; NH), 9.51002 (s; OH), 8.03405-7.33288 (m; Ar- H), 5.30011 (s; NH_2), 1.37415-1.32802 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1786 (CH), 126.1852 (CH), 127.2035 (CH), 131.2606 (C), 133.5505 (C), 138.5682 (CH), 148.4652 (C), 30.6089 (CH_2), 25.8079 (CH_2), 165.5396 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S_2$) calc. 311.05Da, Found: 311 Da;Calculated for $C_{11}H_{13}N_5O_2S_2$, C, 42.43; H, 4.21; N, 22.49; O, 10.28; S, 20.60 Found: C, 41.99; H, 4.28; N, 30.06; O, 11.39; S, 11.72.

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6g)

To an equimolar mixture of 4-amino-5-(4-methylphenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce yellowish white crystals.

Yield: 1.8 g (80%), mp: 138-140 °C

Analysis: FTIR (KBR) (cm^{-1}): 3343.96 (N-H stretching), 3048.17 (aromatic C-H stretching), 2917.92 (aliphatic C-H stretching), 1443.74 (aromatic C=C stretching), 1667.23 (C=O stretching), 1514.60 (C=N stretching), 1269.08 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.64368 (s; NH), 9.30012 (s; OH), 7.82233-7.25141 (m; Ar- H), 5.60085 (s; NH_2), 1.13647 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 129.0487 (CH), 129.2014 (CH), 129.2701 (CH), 127.9722 (C), 142.9518 (C), 129.3370 (CH), 148.1818 (C), 30.4883 (CH_2), 21.0502 (CH_2), 167.2505 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S$) calc. 279.08 Da, Found: 279 Da; Calculated for $C_{11}H_{13}N_5O_2S$, C, 47.30; H, 4.69; N, 25.07; O, 11.46; S, 11.48 Found: C, 48.10; H, 4.18; N, 29.86; O, 11.14; S, 11.57.

3-((4-amino-5-(p-tolyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6h)

To an equimolar mixture of 4-amino-5-(4-methylphenyl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce pale yellow crystals.

Yield: 1.4 g (73%), mp: 148-150 °C

Analysis: FTIR (KBR) (cm^{-1}): 3342.98 (N-H stretching), 3071.72 (aromatic C-H stretching), 2918.91 (aliphatic C-H stretching), 1444.07 (aromatic C=C stretching), 1667.44 (C=O stretching), 1515.06 (C=N stretching), 1281.37 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.67881 (s; NH), 9.37850 (s; OH), 7.82335-7.25908 (m; Ar- H), 5.59985 (s; NH_2), 1.16979-1.12207 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 129.0512 (CH), 129.2045 (CH), 129.2723 (CH), 127.9750 (C), 142.9543 (C), 129.3390 (CH), 148.1827 (C), 30.4859

(CH_2), 25.8094 (CH_2), 21.0517 (CH_3), 167.2530 (C). Mass: [EI+] ($C_{12}H_{15}N_5O_2S$) calc. 293.09 Da, Found: 293 Da; Calculated for $C_{12}H_{15}N_5O_2S$, C, 49.13; H, 5.15; N, 23.87; O, 10.91; S, 10.93 Found: C, 48.98; H, 5.10; N, 29.95; O, 10.97; S, 10.99.

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6i)

To an equimolar mixture of 4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.0 g (67%), mp: 223-225°C

Analysis: FTIR (KBR) (cm^{-1}): 3351.84 (N-H stretching), 3070.84 (aromatic C-H stretching), 2939.00 (aliphatic C-H stretching), 1451.03 (aromatic C=C stretching), 1698.61 (C=O stretching), 1572.72 (C=N stretching), 1288.70 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0948 (s; NH), 9.48836 (s; OH), 8.31648-7.97074 (m; Ar- H), 5.49898 (s; NH_2), 1.19998 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0874 (CH), 128.4884 (CH), 136.8090 (C), 147.9985 (C), 148.7815 (C), 30.6092 (CH_2), 167.2478 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, Found: 310 Da;Calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 Found: C, 39.76; H, 2.95; N, 26.73; O, 20.48; S, 10.13

3-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6j)

To an equimolar mixture of 4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and

extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.2 g (71%), mp: 212-215°C

Analysis: FTIR (KBR) (cm^{-1}): 3365.73 (N-H stretching), 3076.11 (aromatic C-H stretching), 2857.26 (aliphatic C-H stretching), 1475.39 (aromatic C=C stretching), 1681.59 (C=O stretching), 1519.24 (C=N stretching), 1311.25 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1975 (s; NH), 9.15008 (s; OH), 8.32100-7.98191 (m; Ar- H), 5.48758 (s; NH_2), 1.20831-1.16040 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0878 (CH), 128.4887 (CH), 136.8098 (C), 147.9989 (C), 148.7818 (C), 30.6095 (CH_2), 25.8370 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, Found: 324 Da; Calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 Found: C, 41.14; H, 3.58; N, 26.06; O, 19.95; S, 10.07.

2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6k)

To an equimolar mixture of 4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxyacetamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1g (70%), mp: 220-224 °C

Analysis: FTIR (KBR) (cm^{-1}): 3308.80 (N-H stretching), 3082.67 (aromatic C-H stretching), 2926.24 (aliphatic C-H stretching), 1481.03 (aromatic C=C stretching), 1700.90 (C=O stretching), 1518.48 (C=N stretching), 1296.15 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300

MHz) δ ppm: 14.1101 (s; NH), 9.4886 (s; OH), 8.9514-7.8044 (m; Ar- H), 5.8517 (s; NH_2), 1.2327 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0683 (CH), 131.2639 (CH), 132.0087 (C), 133.0016 (CH), 148.0011 (C), 148.9088 (C), 31.8313 (CH_2), 165.7338 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, Found: 310 Da; Calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 Found: C, 38.80; H, 3.29; N, 26.98; O, 20.61; S, 10.30.

3-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6l)

To an equimolar mixture of 4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazole-3-thiol and 3-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20ml water was added and extracted with chloroform thrice, aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Yield: 1.1 g (70%), mp: 210-212 °C

Analysis: FTIR (KBR) (cm^{-1}): 3388.56 (N-H stretching), 3097.85 (aromatic C-H stretching), 2904.84 (aliphatic C-H stretching), 1460.72 (aromatic C=C stretching), 1695.40 (C=O stretching), 1513.61 (C=N stretching), 1285.51 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1199 (s; NH), 9.4883 (s; OH), 8.9488-7.1773 (m; Ar- H), 5.8455 (s; NH_2), 2.2546 (s; CH_2), 1.2186 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0678 (CH), 131.2635 (CH), 132.0088 (C), 133.0010 (CH), 148.0003 (C), 148.9085 (C), 31.8309 (CH_2), 25.9689 (CH2), 165.7335 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, Found: 324 Da;Calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 Found: C, 40.64; H, 3.67; N, 25.99; O, 19.79; S, 9.92.

4.1. SPECTRAL DATA

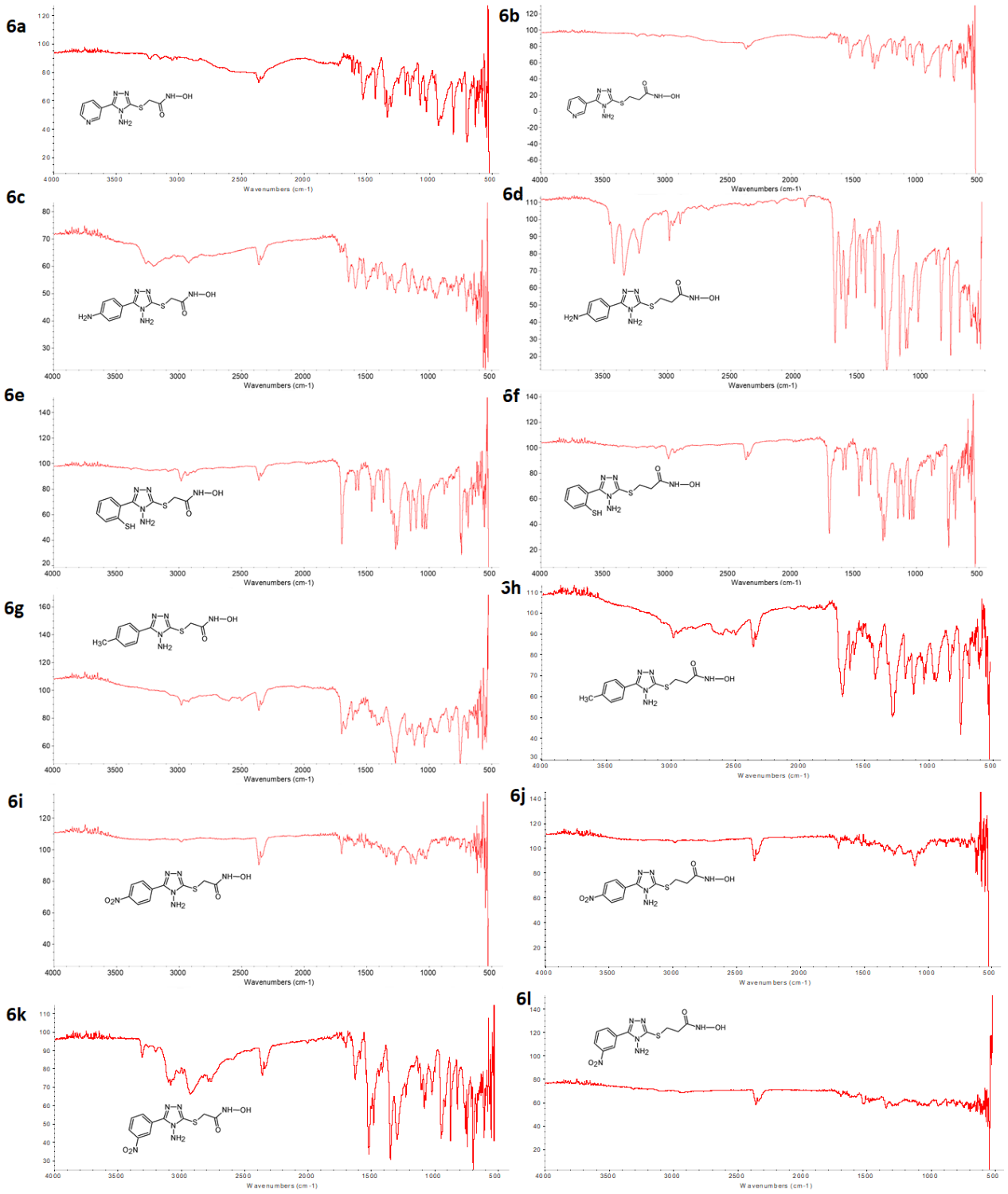


Fig 8. FTIR spectra of 6a, 6b, 6c, 6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l

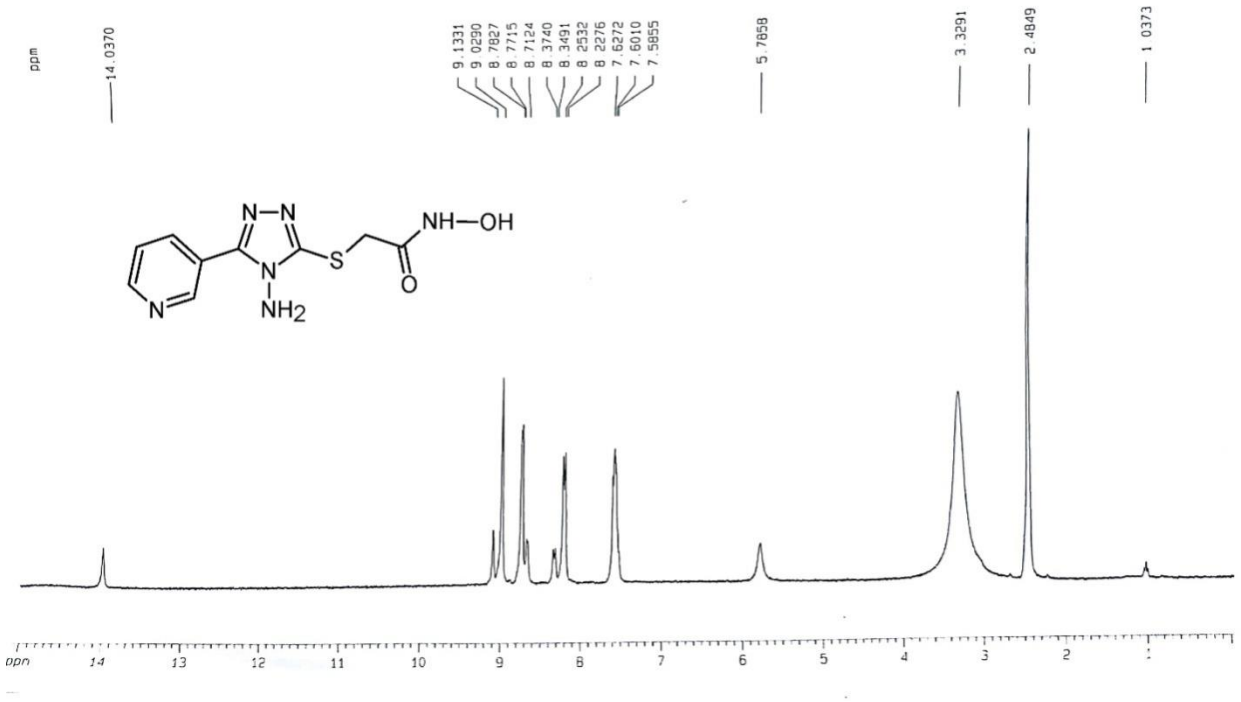


Fig 9. ^{1}H NMR spectrum of compound 6a in DMSO-*d6*

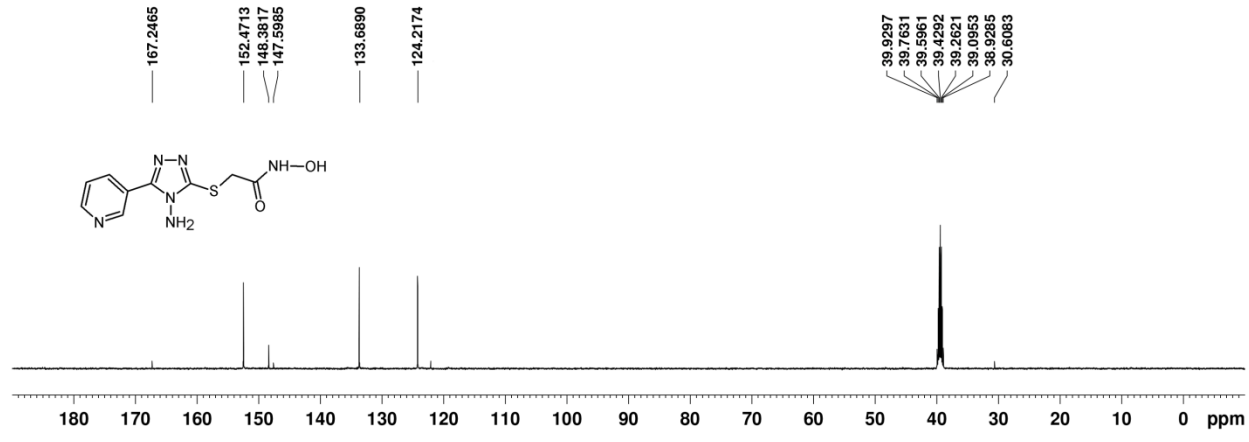


Fig 10. ^{13}C NMR spectrum of compound 6a in DMSO-*d6*

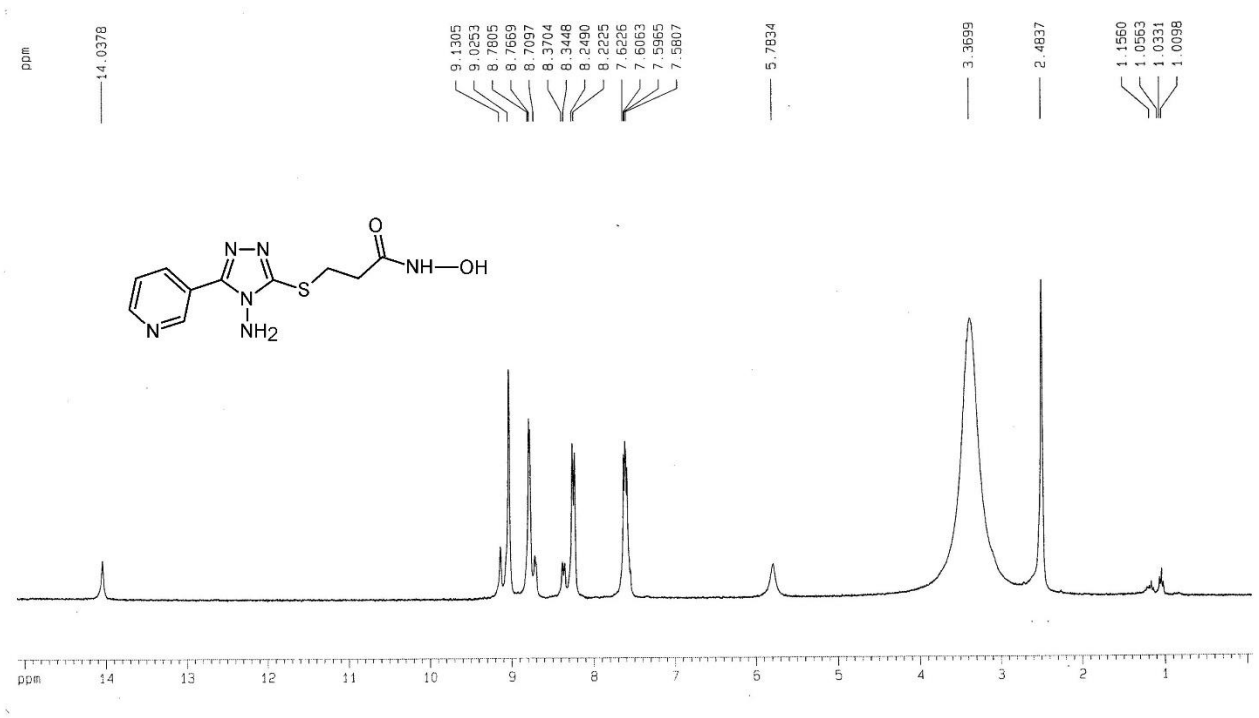


Fig 11. ^{1}H NMR spectrum of compound 6b in DMSO-*d6*

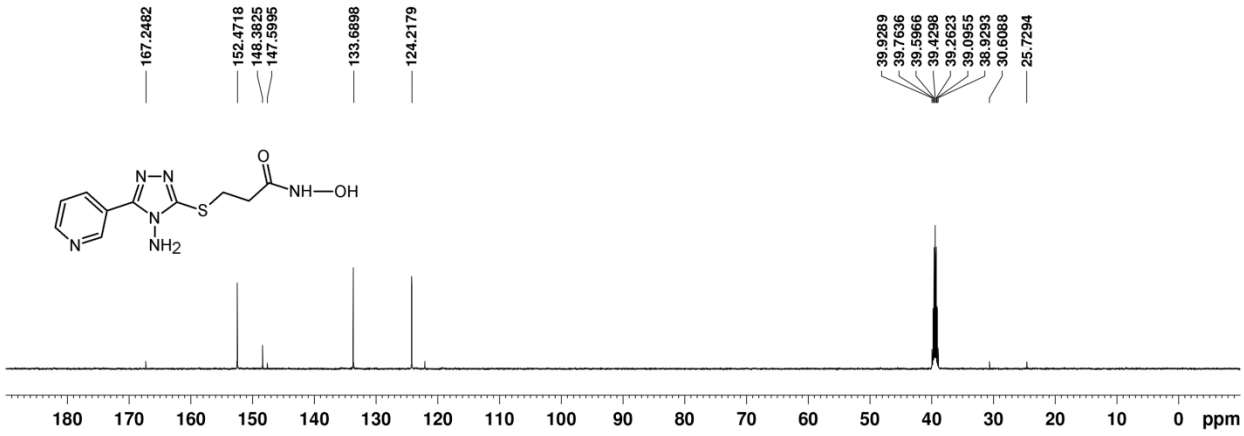


Fig 12. ^{13}C NMR spectrum of compound 6b in DMSO-*d6*

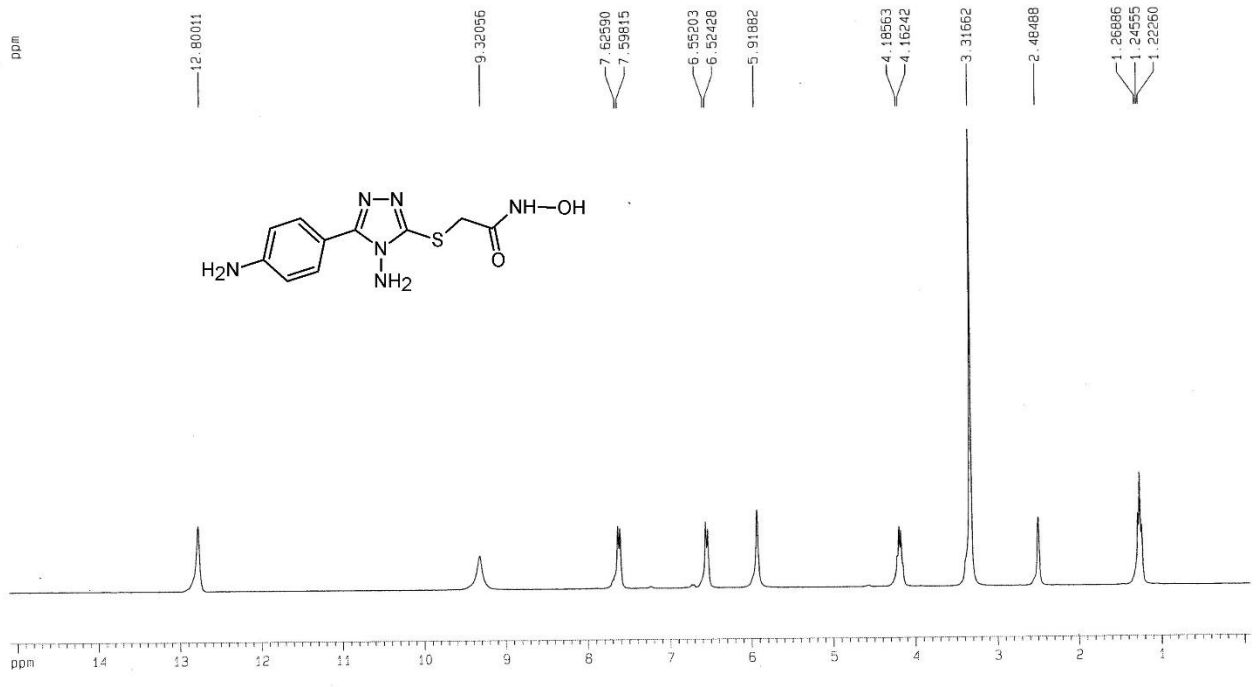


Fig 13. ^{1}H NMR spectrum of compound 6c in DMSO-*d6*

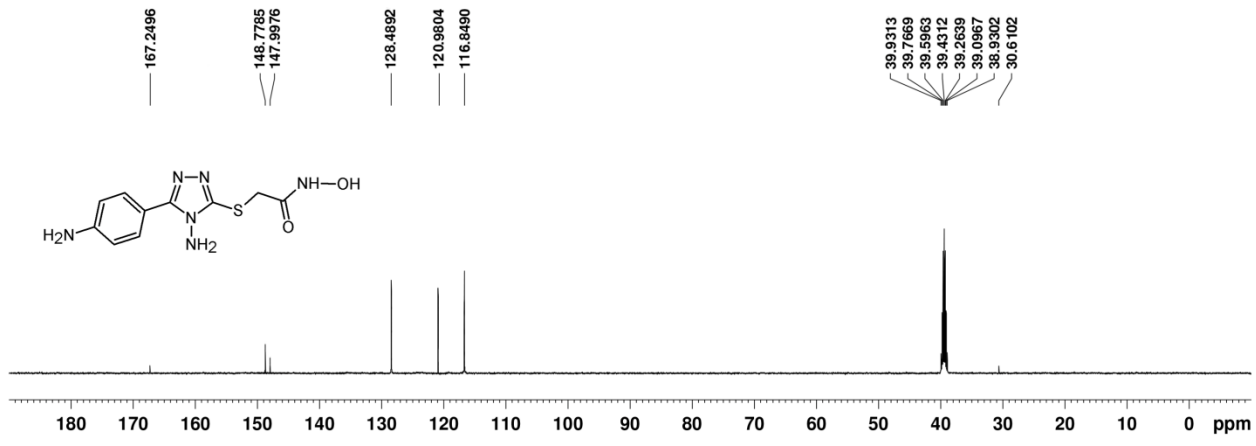


Fig 14. ^{13}C NMR spectrum of compound 6c in DMSO-*d6*

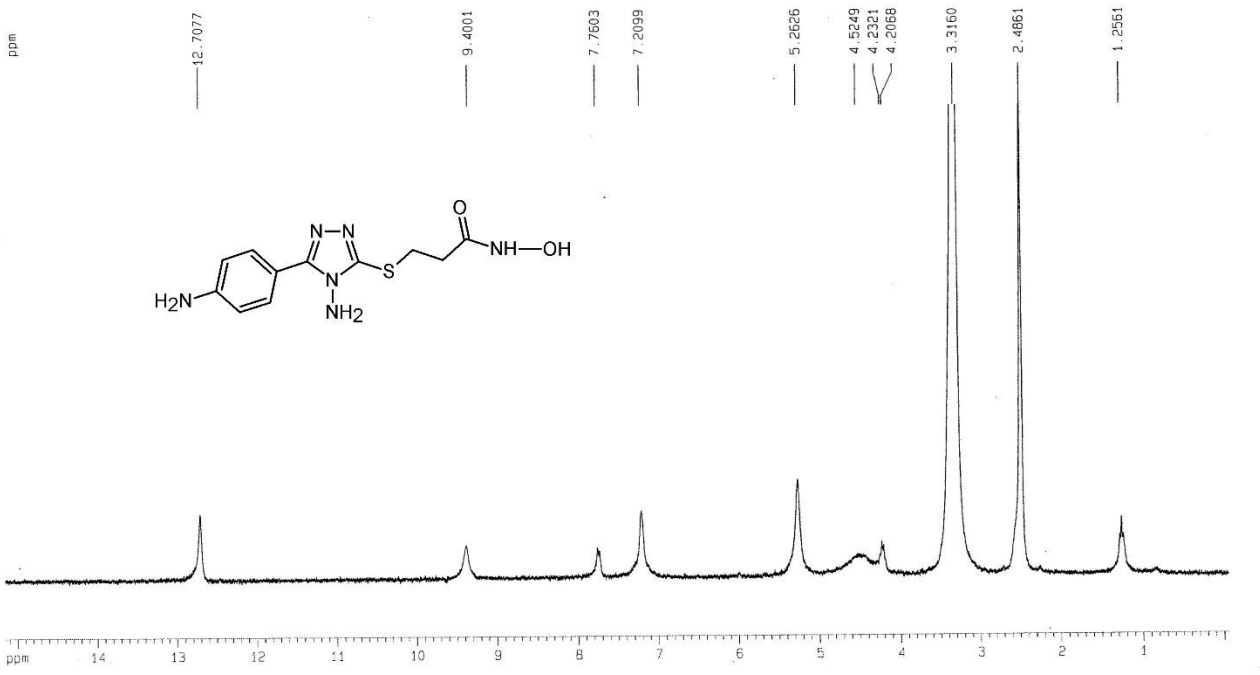


Fig 15. ^{1}H NMR spectrum of compound 6d in DMSO-*d6*

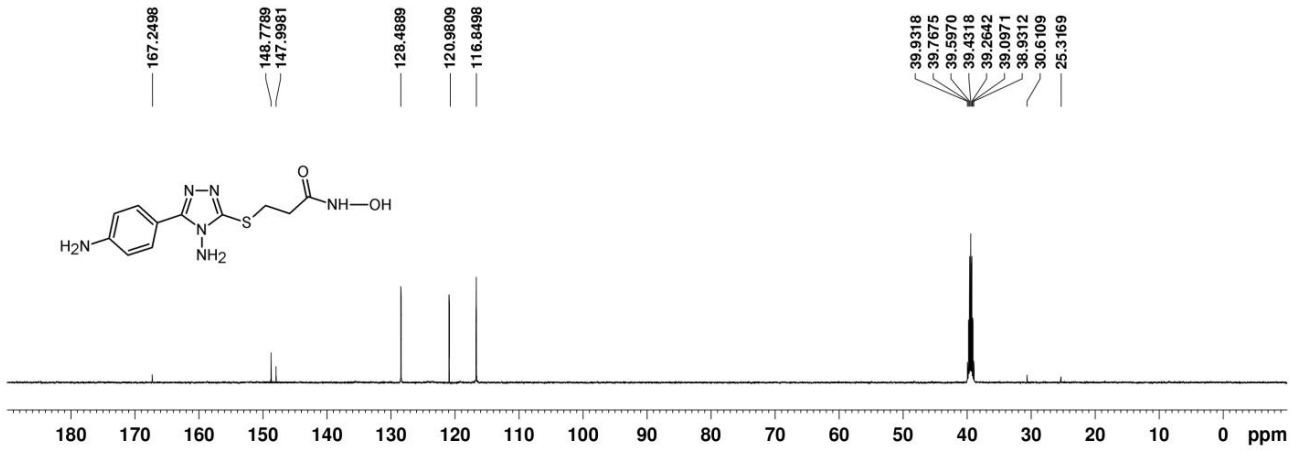


Fig 16. ^{13}C NMR spectrum of compound 6d in DMSO-*d6*

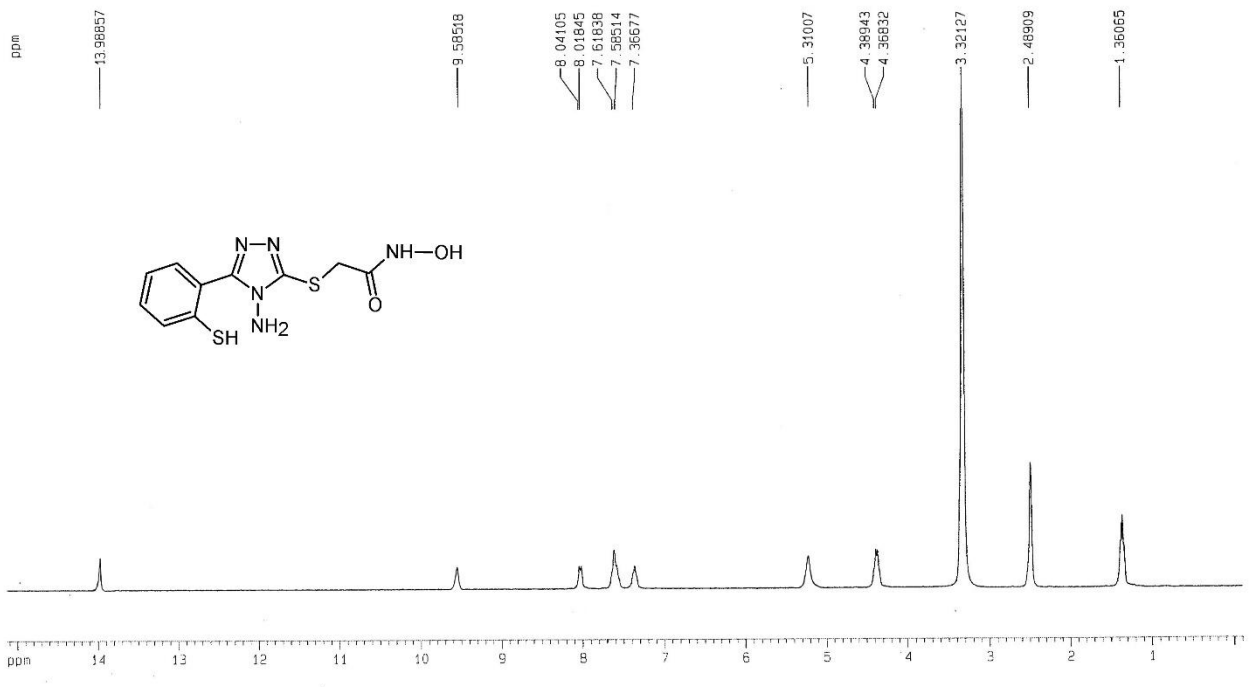


Fig 17. ^{1}H NMR spectrum of compound 6e in DMSO-*d6*

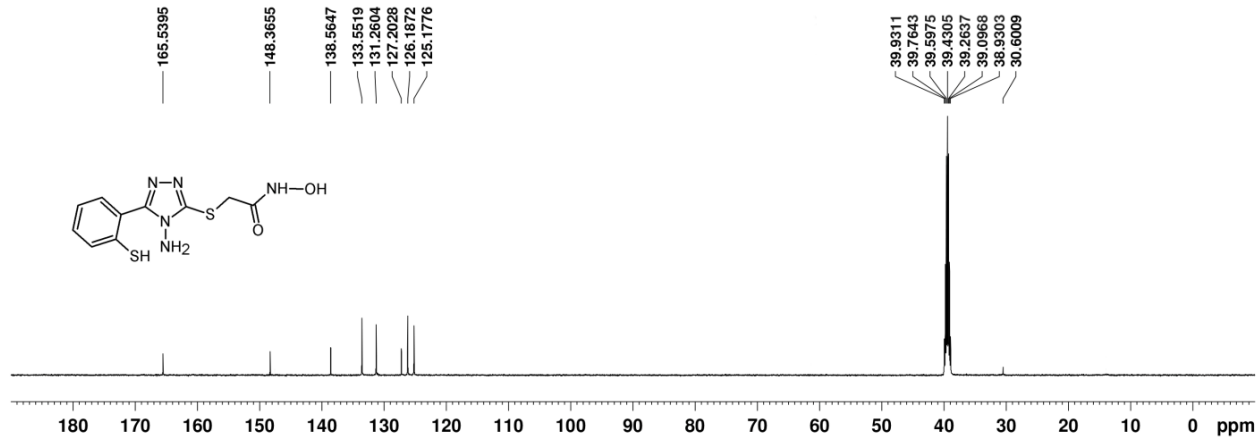


Fig 18. ^{13}C NMR spectrum of compound 6e in DMSO-*d6*

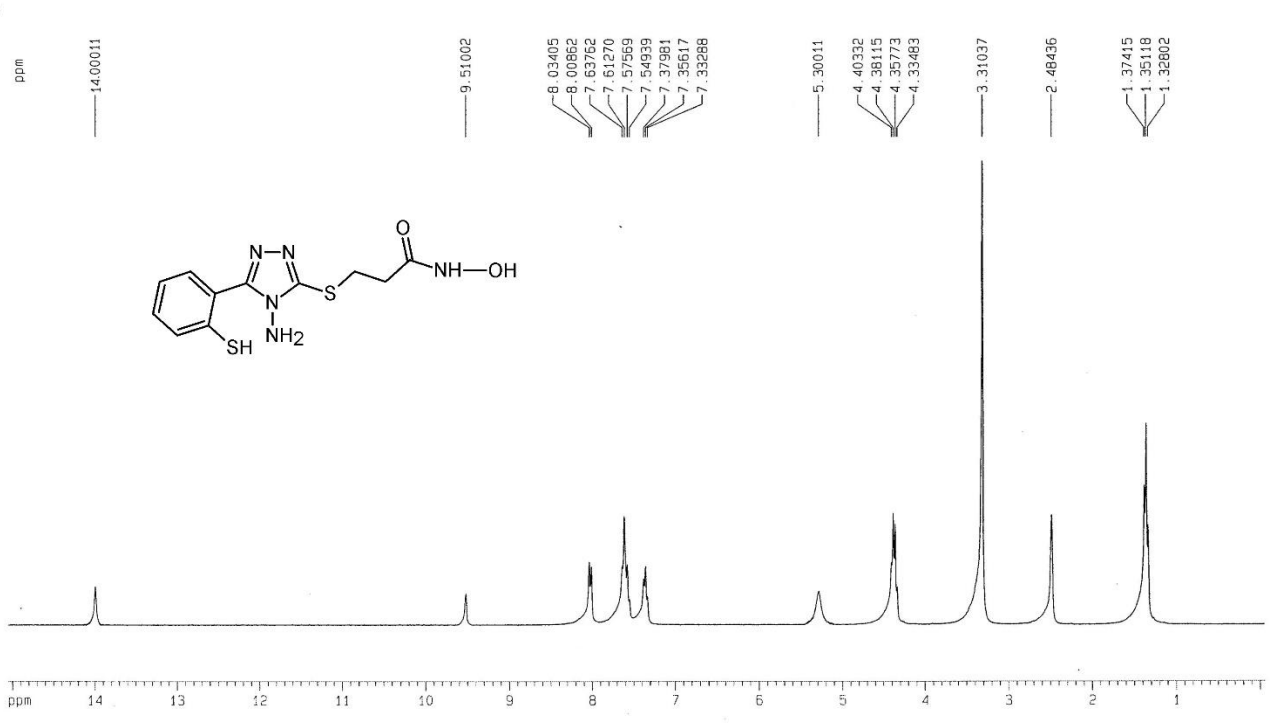


Fig 19. ^{1}H NMR spectrum of compound 6f in DMSO-*d6*

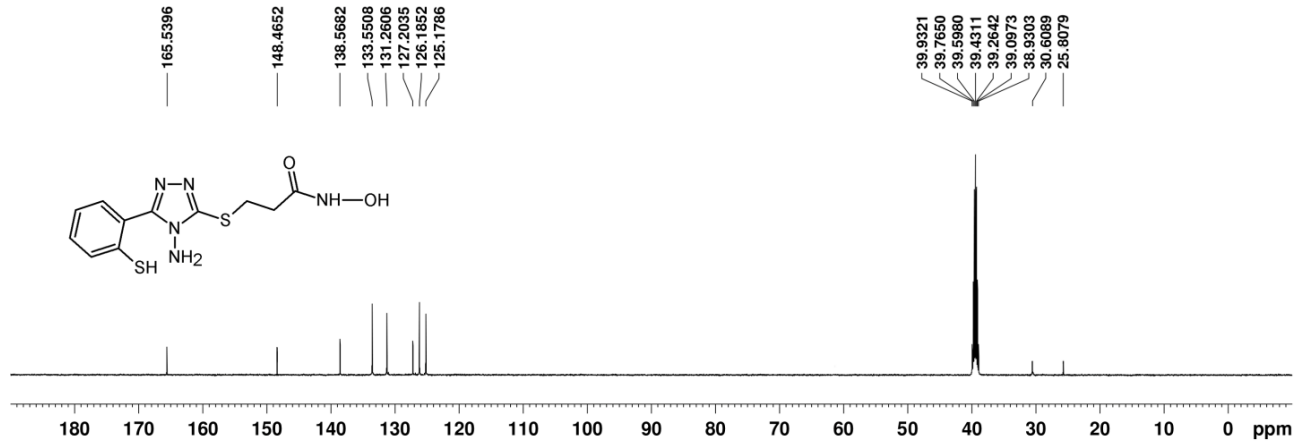


Fig 20. ^{13}C NMR spectrum of compound 6f in DMSO-*d6*

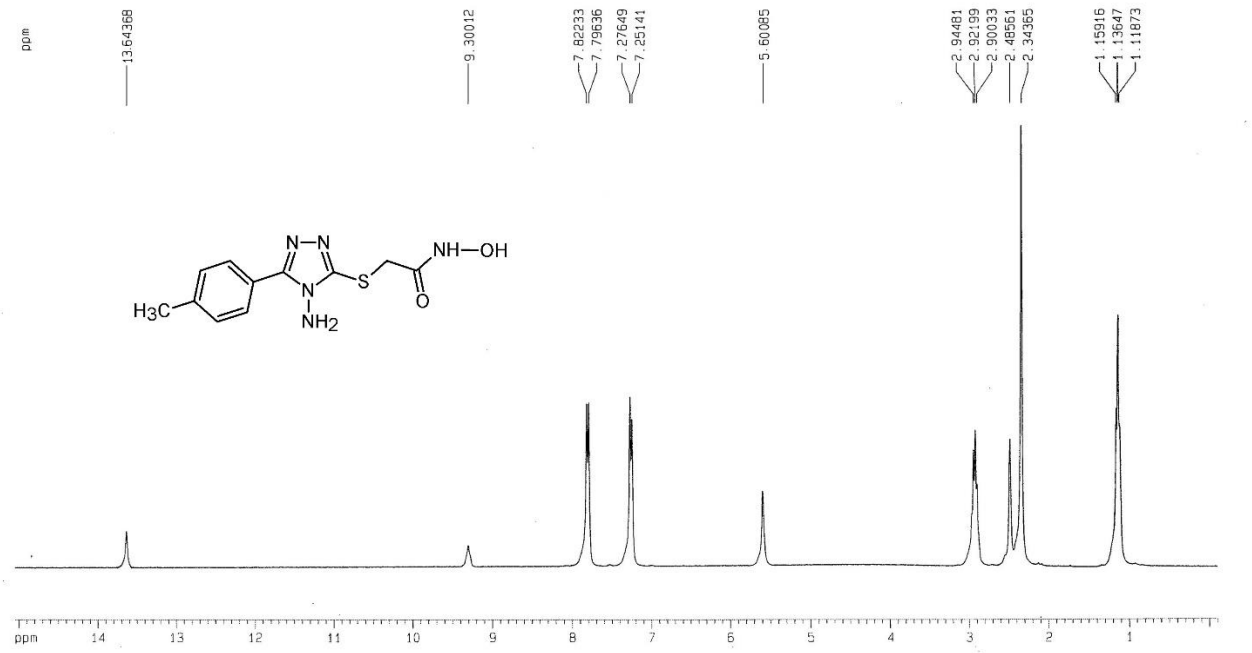


Fig 21. ^{1}H NMR spectrum of compound 6g in DMSO-*d6*

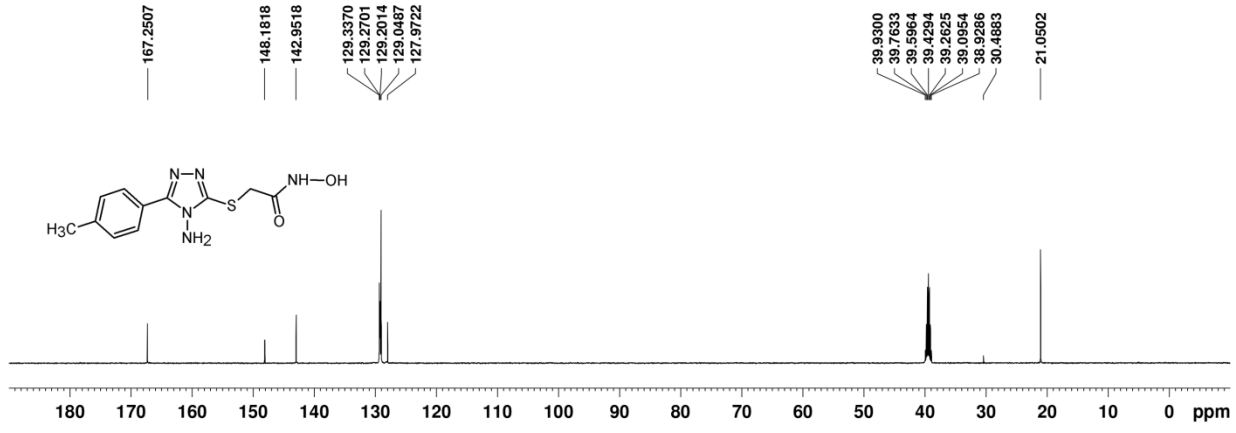


Fig 22. ^{13}C NMR spectrum of compound 6g in DMSO-*d6*

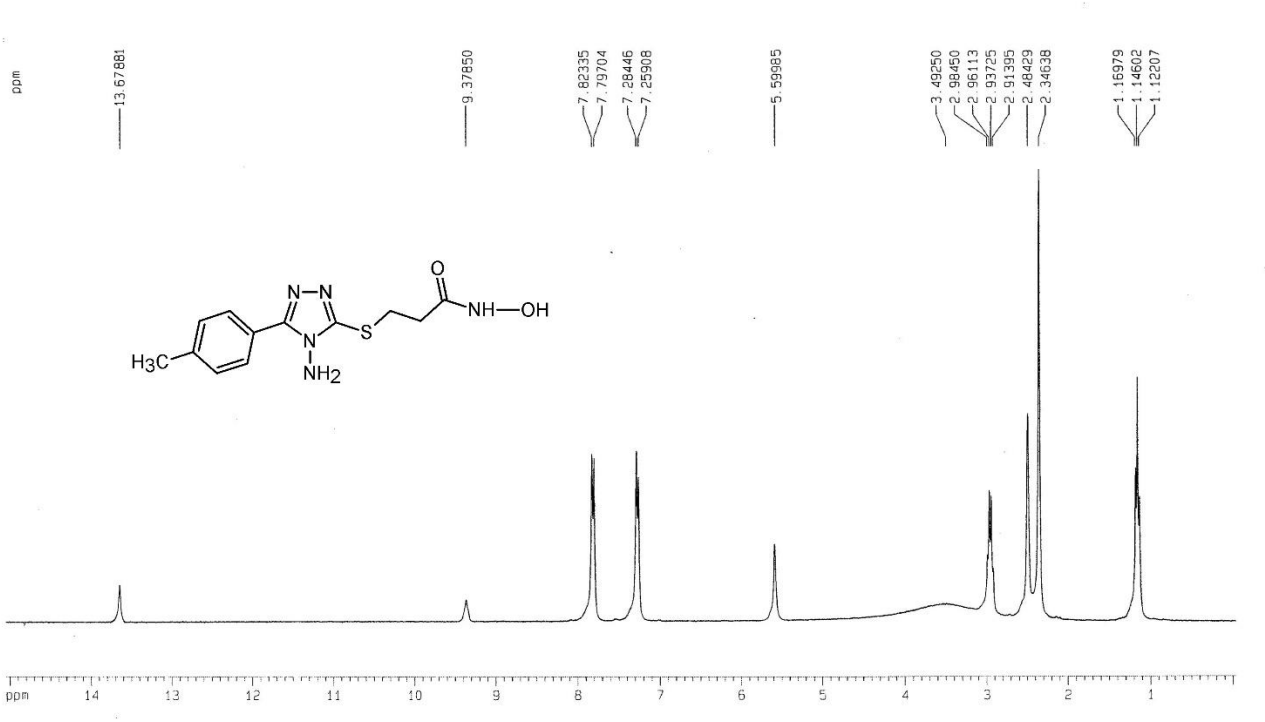


Fig 23. ^{1}H NMR spectrum of compound 6h in DMSO-*d6*

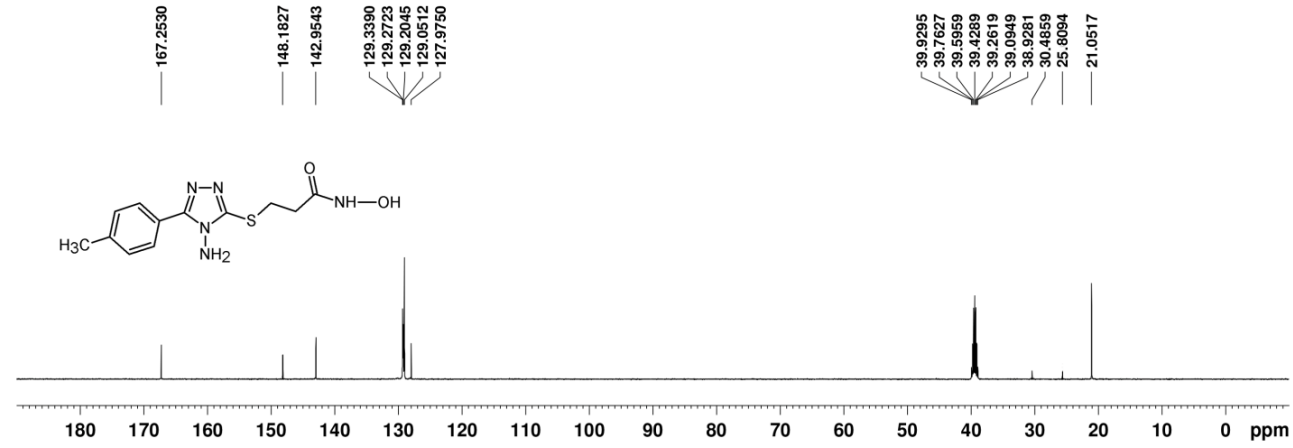


Fig 24. ^{13}C NMR spectrum of compound 6h in DMSO-*d6*

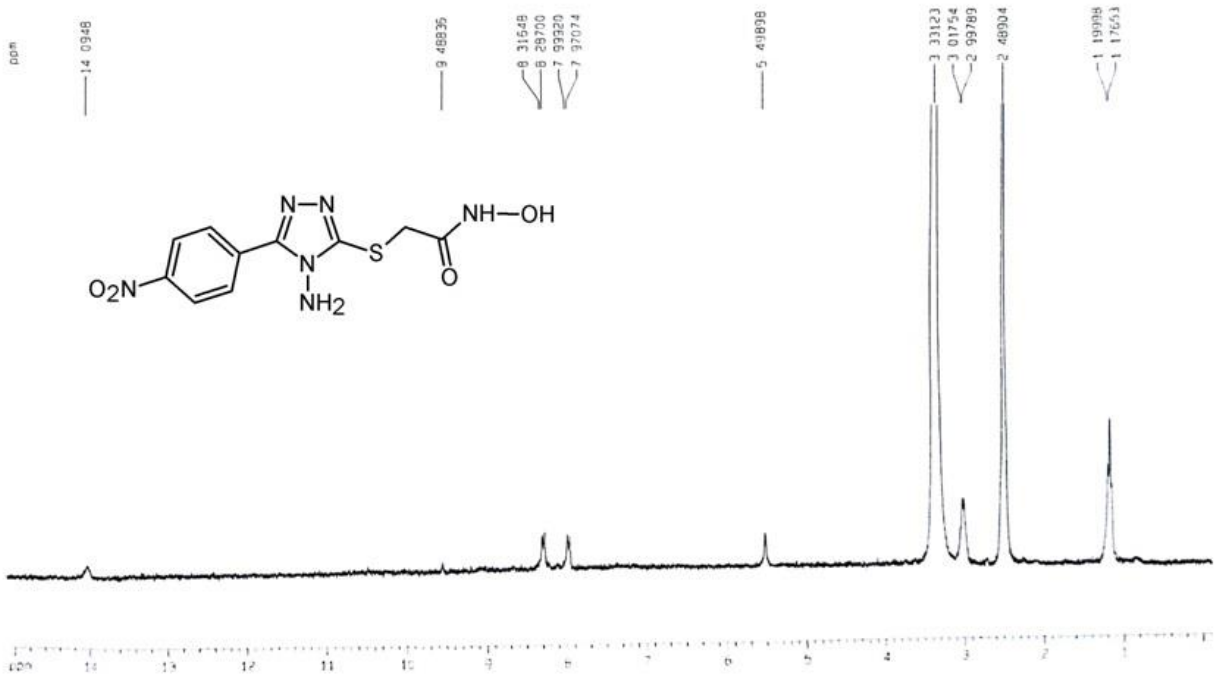


Fig 25. ^{1}H NMR spectrum of compound 6i in DMSO-*d6*

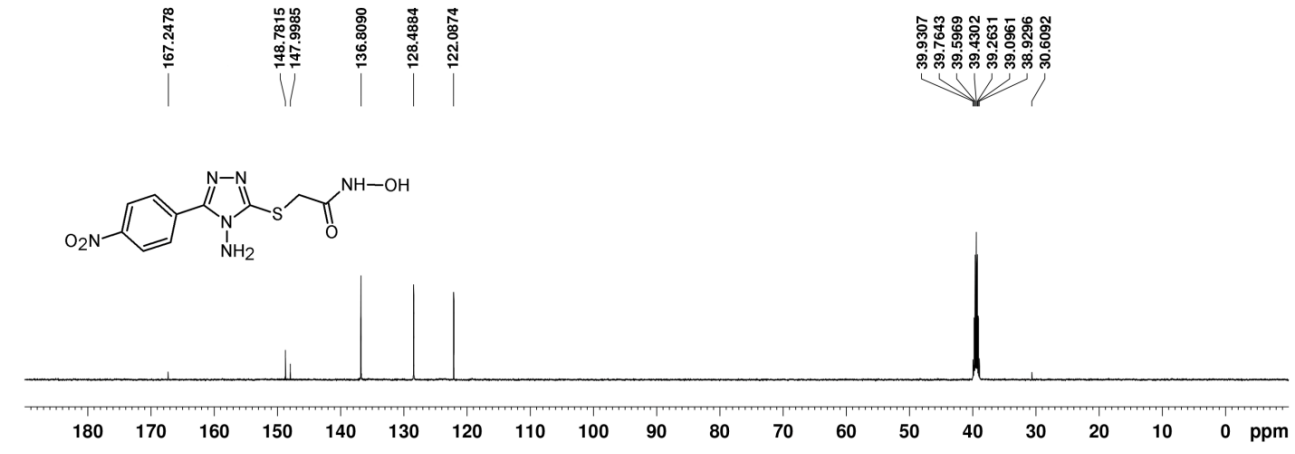


Fig 26. ^{13}C NMR spectrum of compound 6i in DMSO-*d6*

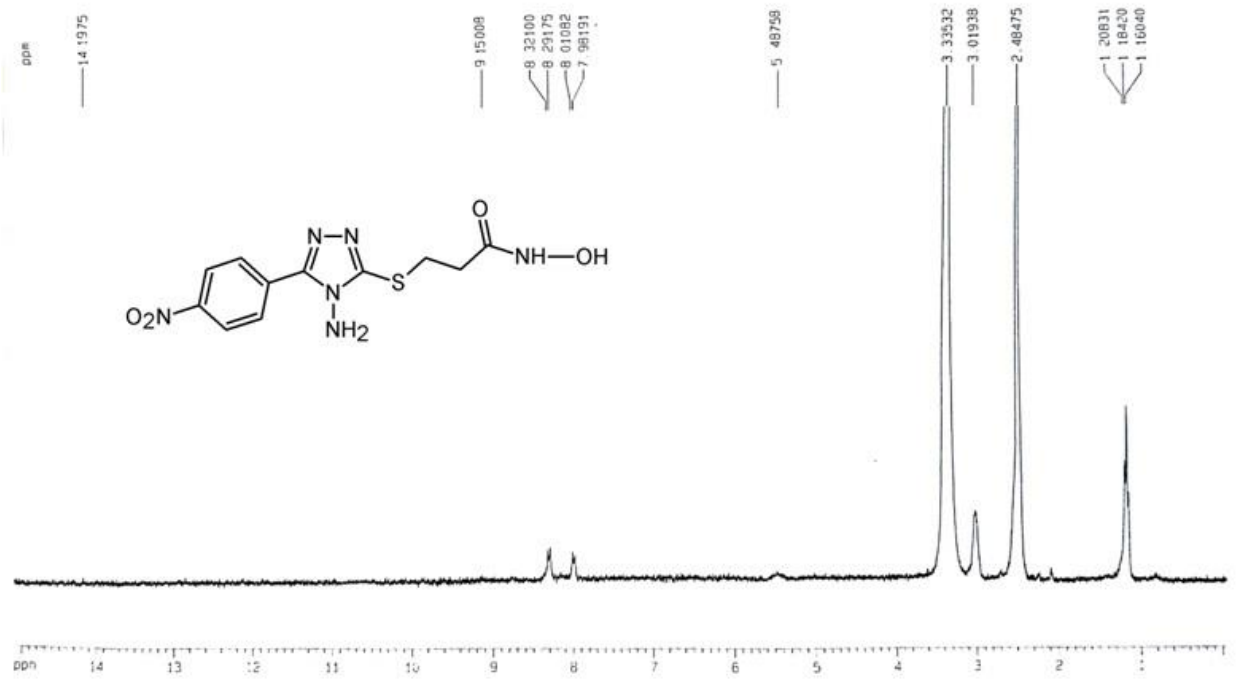


Fig 27. ^{1}H NMR spectrum of compound 6j in DMSO-*d6*

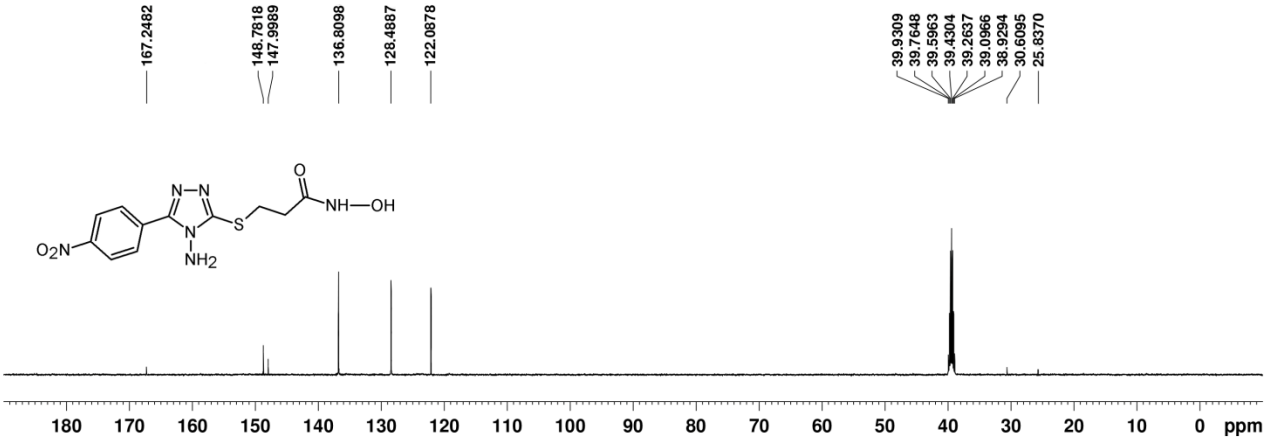


Fig 28. ^{13}C NMR spectrum of compound 6j in DMSO-*d6*

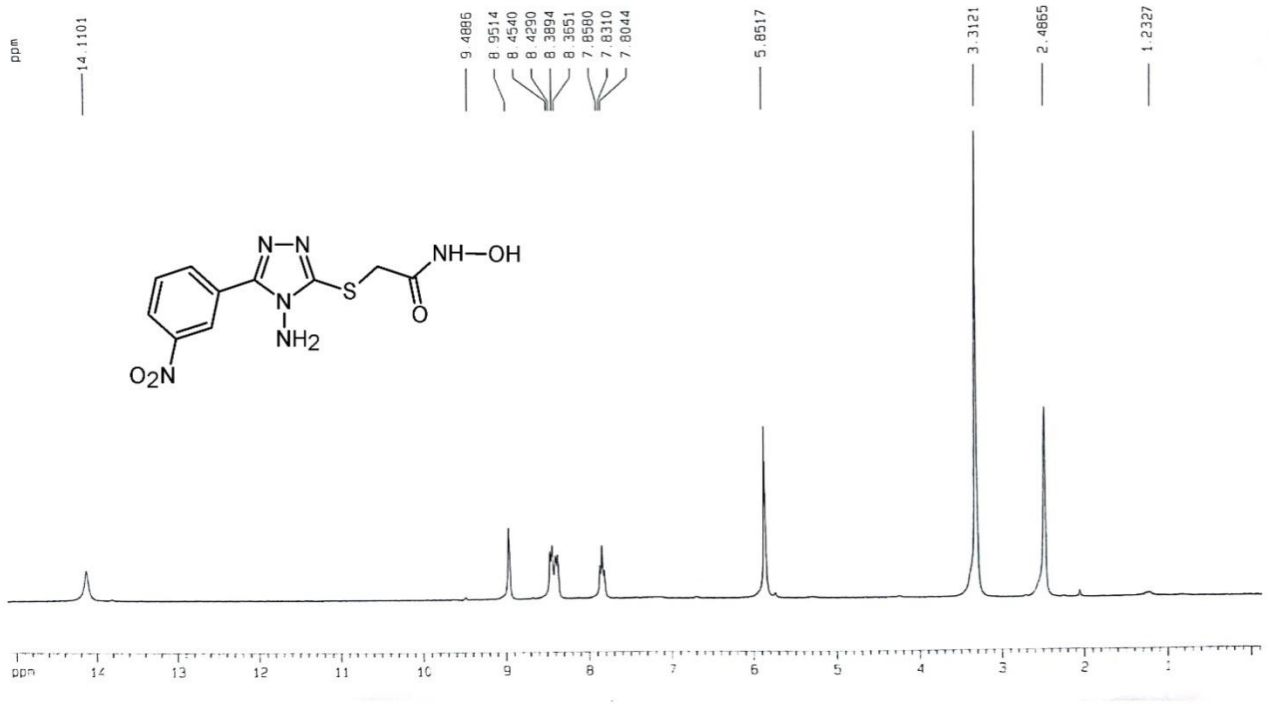


Fig 29. ^{1}H NMR spectrum of compound 6k in DMSO-*d6*

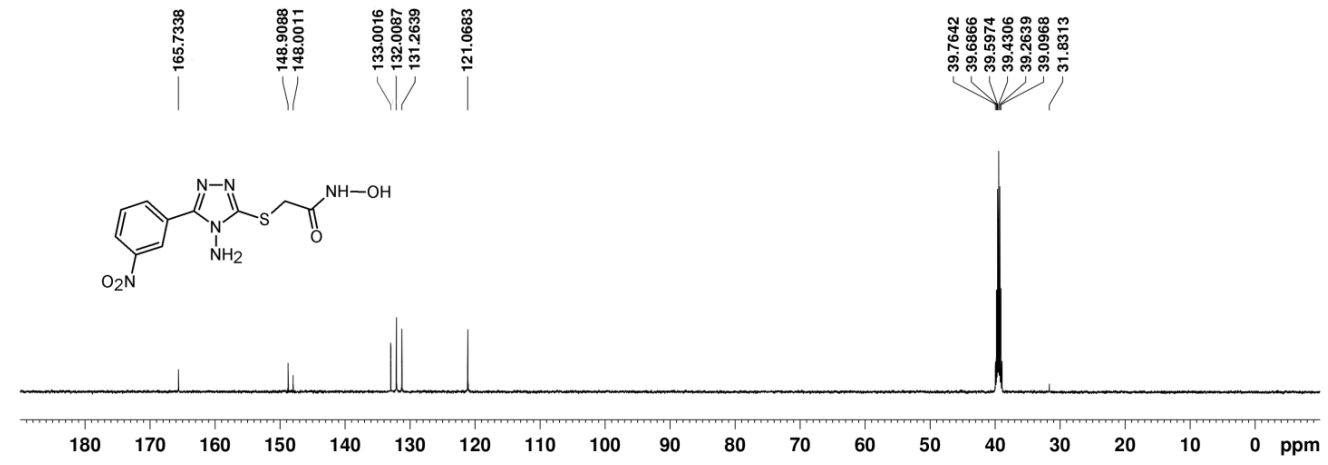


Fig 30. ^{13}C NMR spectrum of compound 6k in DMSO-*d6*

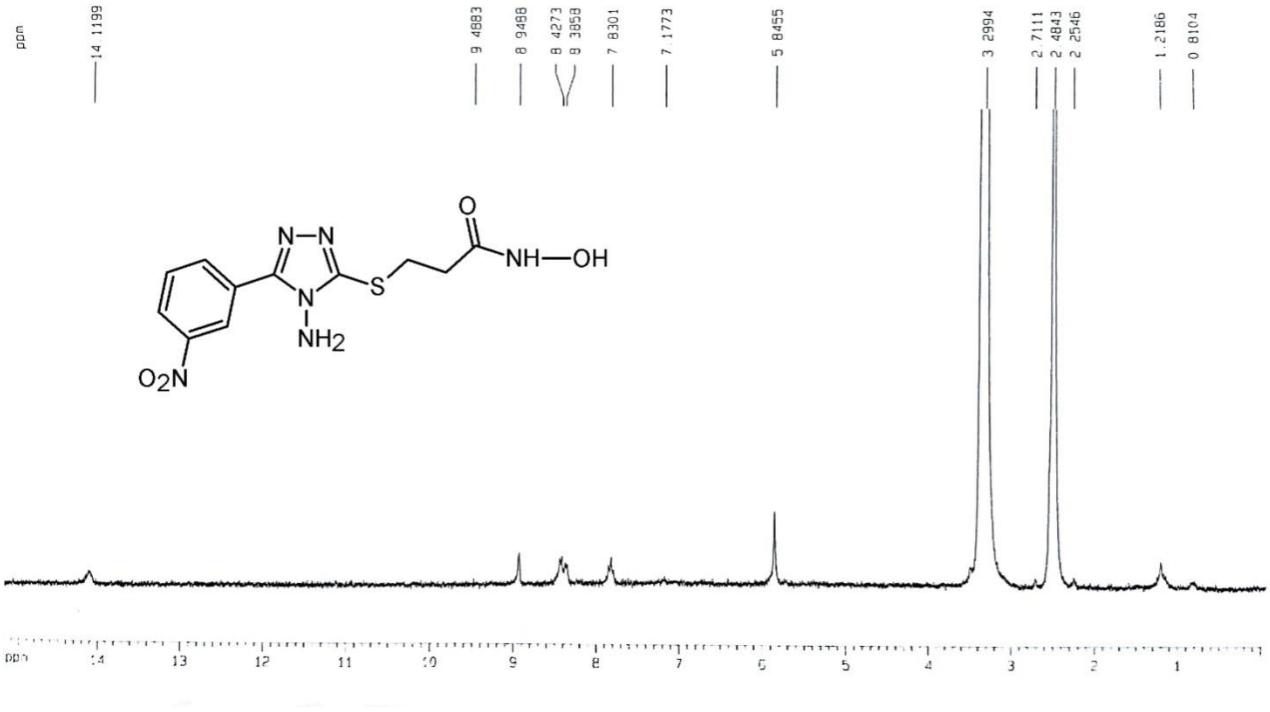


Fig 31. ^{1}H NMR spectrum of compound 6l in DMSO-*d6*

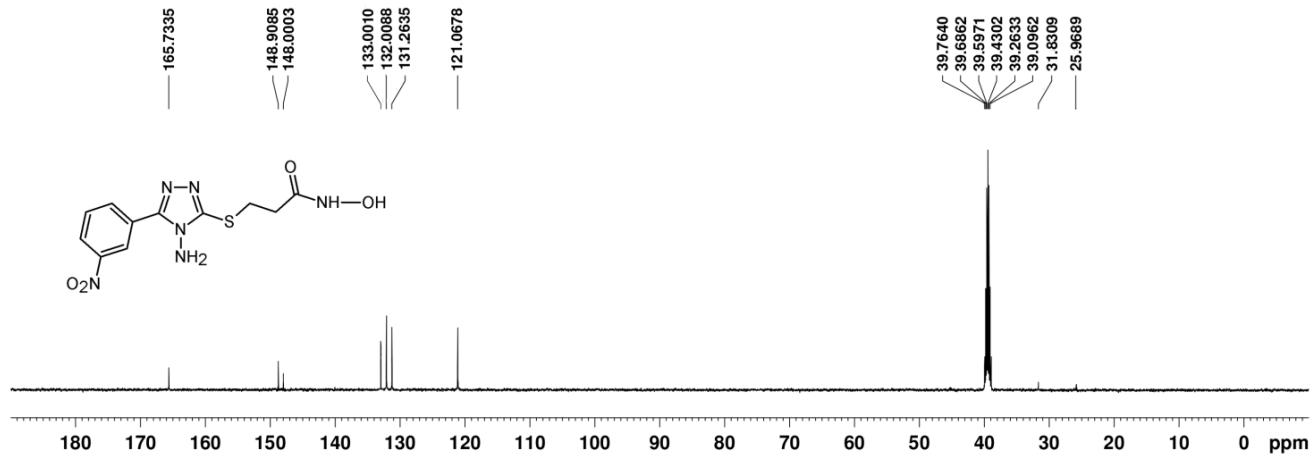


Fig 32. ^{13}C NMR spectrum of compound 6l in DMSO-*d6*

Chapter 5

5. Antimicrobial study

The Microbes are getting resistant towards the existing chemotherapeutics in alarming rate which is not only a major concern for public health but also a challenge for the scientific community globally and the number of cases of multidrug-resistant bacterial infections is increasing now a day (Shalini et al., 2011). In clinical practice, the infections caused by the gram-positive bacteria are very common. However, the infection caused by them is sometimes severe. *Bacillus cereus, Staphylococcus aureus, Bordetella bronchiseptica*, *Micrococcus luteus*, *Bacillus pumilus, Bacillus subtilis, Enterococcus faecalis* are some important gram-positive bacteria causing serious infection in community. Whereas, *Salmonella typhi*, *Klebsiella pneumoniae, Vibrio cholerae, Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* are very common gram-negative bacteria. Among them, *Escherichia coli* is the most common one. The mortality is higher for infections caused by gram-negative bacteria than the gram-positive one. To overcome various microbial infections specifically fungal infection, a large number of triazole drugs have been successfully developed (Gadhave et al., 2010). Azole compounds showed the effect by inhibiting lanosterol 14α-demethylase (CYP51) which catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis (Aoyama et al., 1984) resulting in accumulation of toxic methyl-sterols in membranes leading to fungistatic or fungicidal effect (Kelly et al., 1993). Some important Azoles derivatives like voriconazole, itraconazole, posaconazole, fluconazole are used for the treatment of fast-growing fungal infections, which are the leading cause of mortality and morbidity in immuno-compromised patients (Groll et al., 2012). However, Fluconazole is not effective against aspergillosis because of its severe drug resistance (Casalinuovo et al., 2004 and Hoffman et al., 2000).

These scenarios are highlighting the urgent need for novel, efficacious, less toxic and safe drug candidates in the pipeline. The heterocyclic compounds, containing nitrogen, sulfur and oxygen have an enormous significance in the field of medicinal chemistry (Crumplin et al., 1975). To address the concern, we develop a series of novel 5-substituted-4-amino-1,2,4-triazole- linked hydroxamic acid derivatives. Assembling the different pharmacophores in a single frame always leads to compounds having a fascinating biological profile (Aggarwal et al., 2010). Triazole is one of the pharmacophores in the synthesized compounds. Chemical compounds possess triazole as a core; always play a significant role in the field of medicinal chemistry. Like triazole, imidazole moiety has widely used to synthesize compounds to treat diseases causing by different microorganisms. Even though imidazole and triazole have a similar mode of action, triazoles have several advantages over imidazole, like, oral bioavailability, slow metabolic rate and have minimal effect on sterol synthesis in humans. So, the use of triazole moiety increases over imidazole (Palekar et al., 2009). Triazole is well known for its antimicrobial property and widely used to treat microorganisms associated diseases (Jain et al., 2013). 1,2,4-triazoles derivatives are more stable in structure and shown anti-tubercular, hypoglycemic, anticancer, analgesic and antidepressant activities Besides the antibacterial (Prasad et al., 2009; Foroumadi et al., 2003; Ram et al., 1990 and Ergenc et al., 1992) and antifungal (Ram et al., 1974; Reddy et al., 1990; Hiremath et al., 1989 and Kalyoncuoğlu et al., 1992) activity; 1,2,4-triazole possess, anti-inflammatory (Sarigol et al., 2015) as well as antioxidants properties (Yehye et al., 2016). Various well-known drug commercialized in the market possess 1,2,4-triazole moiety like fluconazole, terconazole, itraconazole, (Sztanke et al., 2008 and Haber et al., 2001) triazolam, (Adams et al., 1983) ribavirin (Sidwell et al., 1975) etc. Different S and N-bridged heterocycles can be synthesized by 1,2,4-triazoles as it possesses nucleophilic centers. Triazolothiazines,

triazolothiadiazoles, triazolothiazepines, triazolothiadiazines, and thiazolotriazoles can be synthesized from triazoles, having versatile therapeutic potentials. Another moiety in our synthetically designed compounds is hydroxamic acid. It is another interesting moiety possess antibacterial, antifungal (Indiani et al., 2003 and Pavlaki et al., 2003), anti-inflammatory (Mai et al., 2006 and Hanessian et al., 2003), antitumor (Marks et al., 2007) and anticancer agents (McInnes et al., 2007). Hydroxamic acid and its derivatives can exert versatile activity by inhibiting several enzymes, like, urease (Odake et al., 1994), matrix metalloproteinases (Whittaker et al., 1999 and Wada et al., 2002), ribonucleotide reductase (Nandy et al., 1999), 5-lipoxygenase (Kerdesky et al., 1987) etc. Hydroxamic acid has the ability to chelate with metals. Few ion exchange resins are built on hydroxamic acids (Cocea et al., 1965). A number of hydroxamic acid derivatives are used as chemotherapeutic agents like, ibuproxam, hydroxycarbamide, desferrioxamine B, oxametacin, adrafinil, bufexamac etc (Shiver et al., 2002). So, considering the above fact we have designed and synthesized some novel 5-substituted-4-amino-1,2,4-triazole linked hydroxamic acid derivatives and evaluate their activity against several gram-positive, gram-negative bacteria, a number of fungal strains.

5.1. Materials and methods

5.1.1. Materials

All the chemicals were procured from Sigma-Aldrich, India, Spectrochem, Merck India Pvt. Ltd., and the media were of purchased from Himedia Laboratories. Dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were procured from Merck, India; all other chemicals used were of analytical grade. In process monitoring of reaction was done on activated silica gel coated plates and the solvent system used was n-hexane: ethyl acetate. The melting points of the synthesized compounds were measured by the capillary method and are reported uncorrected. The FT-IR

Spectra were measured in Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA), ^{1}H NMR spectra were recorded on AV300 Digital FT NMR Spectrometer, Bruker at 300 MHz using DMSO-d_6 as the solvent and tetramethylsilane as an internal standard, ^{13}C NMR spectra were obtained at 500 MHz (Bruker, Germany), DMSO-d_6 as the solvent. Mass spectra (m/z) of the compounds were recorded on JEOL-JMS 700 spectrometer using electron ionization technique. Scanning electron microscopy (SEM) study of the synthesized compounds was analyzed using scanning electron microscope JEOL, Tokyo, Japan.

5.1.2. Media

5.1.2.1. Preservation of cultures

All the strains were preserved in stab agar media. Routine sub culturing of the Gram positive bacteria was carried out on nutrient agar and of the Gram negative bacteria on bromothymol blue lactose agar (Sen et al., 1968). Fungal strains were maintained at 4ºC on Sabouraud's glucose agar slants, sub cultured and incubated at 28º-30 ºC before performing antifungal activity tests.

5.1.2.2. Standard Drugs

The reference drugs amoxicillin trihydrate and fluconazole were obtained in pure dry powder form.

5.1.2.3. Bacteriological culture media

The media used for this study were primarily of two types: liquid culture media and solid culture media.

5.1.2.3.1. Liquid culture media

Three types of liquid culture media were used-

- **Nutrient broth**

  This medium constituted of the following ingredients

- Bacteriological peptone 1.0 %
- Beef extract 1.0 %
- Sodium chloride 0.5 %
- pH 7.2-7.4

❖ **Peptone water**

This medium consisted of the following ingredients

- Bacteriological peptone 1.0 %
- Sodium chloride 0.5 %
- pH 7.2-7.4

❖ **Sabouraud's glucose broth**

The composition of this medium was as follows

Glucose	4.0%
Mycological peptone	1.0 %
pH	5.4

5.1.2.3.2. Solid culture media

Different types of solid culture media were used

❖ **Peptone agar**

- Agar 1.0 %
- Bacteriological peptone 1.0 %
- Sodium chloride 0.5 %
- pH 7.2-7.4

❖ **Nutrient agar**

Nutrient agar medium was used as a highly reproducible medium for all the Gram positive bacteria. It consists of the following composition

- Agar 1.5 %
- Beef extract 0.5 %
- Bacteriological peptone 1.0 %
- Sodium chloride 0.5 %
- pH 7.2-7.4

❖ **Bromothymol blue lactose agar**

- Agar 1.5 %
- Beef extract 0.5 %
- Bacteriological peptone 1.0 %
- Sodium chloride 0.5 %
- pH 7.2-7.4

To the above medium (per 100 ml) 1.25 ml of a 0.2% solution of bromothymol blue indicator was added. The pH was adjusted to 7.4. After sterilization at 15 lbs/sq. inch pressure for 15 minutes, 1.0% lactose was added aseptically and the medium was steamed for another 30 minutes before being distributed on Petri dishes (Collee et al., 1996).

❖ **MacConkey's agar**

The composition of this medium was as follows:

- Agar 2.0 g
- Sodium taurocholate, (commercial) 0.5 g
- Bacteriological peptone 2.0 g

- Water 100.0 ml
- Neutral red solution (2% in 50% ethanol) about 0.35 ml
- Lactose, 10% aqueous solution 10.0 ml
- pH 7.5.

❖ **Sabouraud's glucose agar**

The composition of this medium was as follows:

- Glucose 4.0%
- Mycological peptone 1.0 %
- Agar 2.0%
- pH 5.4

5.1.2.4. Microbial strains

In vitro antimicrobial activity was evaluated against 15 different pathogenic bacteria includes both Gram-negative like *Salmonella typhi*62, *Klebsiella pneumoniae* ATCC 10031, *Vibrio cholerae* VC 20, *Pseudomonas aeruginosa* 25619, *Shigella sonnei* NK 4010, *Escherichia coli* ATCC 25923, *Salmonella typhimurium* NTCC 74, and Gram-positive like *Bacillus polymyxa* 4747, *Bacillus cereus* 479, *Staphylococcus aureus* ATCC 29737, *Enterococcus faecalis* 28, *Bordetella bronchiseptica* 4617, *Micrococcus luteus* 10240, *Bacillus pumilus* 148884, *Bacillus subtilis* 6673 respectively, and 7 different fungi like *Candida albicans* MTCC 183, *Candida tropicalis* MTCC 2795, *Cryptococcus neoformans*, *Aspergillus niger* MTCC 281, *Microsporum gypseum*, *Penicillium chrysogenum*, *Cladosporium sp*. All these microbial strains were collected from Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

5.1.2.5. Preparation of Bacterial inoculums

The nutrient broth was used to grow the bacterial strains at 37°C for 24h. Afterward, the bacterial strains were compared to 0.5 McFarland standards (McFarland J., 1907) and standardized the dilution with saline or broth to get a density visually which was equivalent to the 0.5 McFarland standard (McFarland, 1907; a turbidity standard prepared by adding 0.5 ml of 1% barium chloride solution to 99.5 ml of 1% H_2SO_4) and made the dilution with sterile normal saline was so that produced an initial suspension of 2×10^6 CFU/mL for further use. The plate was inoculated within 15 minutes of diluting the broth cultures, so that the standardization was maintained throughout the whole study.

5.1.2.6. Preparation of Fungal inoculums

Czapek-Dox media was used to culture the fungal strains at 28-30°C for 24-72h. The fungal suspension was compared to 0.5 McFarland standards (McFarland J., 1907) and made the dilution with sterile normal saline was so that produced an initial suspension of 2×10^6 CFU/mL for further use.

5.1.2.7. Determination of Minimum Inhibitory Concentration (MIC) of the synthesized compounds against bacterial strains

To determine the MIC of the synthesized compounds agar dilution method was employed as per NCCLS (National Committee for Clinical Laboratory Standards) 2006 protocol (CLSI 2012). The synthesized compounds were initially dissolved in a suitable solvent and then serial dilution was made to obtain the concentrations 10, 25, 50, 75, 100, 150, 200, 250, 350 and 400 µg/mL of nutrient agar; control petri dish was prepared with 4% v/v DMSO. The final pH of all the media were adjusted to 7.2 to 7.4 before pouring into sterile Petri dishes. Bacterial inoculums were prepared from peptone water culture. On the separate agar plates with different drug

concentrations one loopful (internal diameter 2 mm) inoculums (2×10^6 CFU/ml) were spotted and incubated for 24h at 37°C and examined for growth up to 72 h (NCCLS, 2003). The test was performed in triplicate for each organism and the experiment was repeated when necessary. MIC was recorded for any lowest concentration where complete growth inhibition of microorganisms was observed visually.

Amoxicillin trihydrate was used as a reference standard. A solution of amoxicillin trihydrate (0.5 mg /ml) in sterile water was prepared. The solution was added to nutrient agar at 45ºC in varied final concentrations of amoxicillin as follows: 0 (control), 0.5, 1, 2.5, 5, 10, 20, 25, 30, 40, 50 µg per ml of nutrient agar. The final pH of all the media were adjusted to 7.2 to 7.4 before pouring into sterile Petri dishes. The MIC was determined in the same way as described for the synthesized compounds.

5.1.2.8. Determination of minimum inhibitory concentrations (MICs) of the synthesized compounds against fungal strains

The fungal strains were grown in Czapek-Dox media, The synthesized compounds were initially dissolved in a suitable solvent and then serial dilution was made to obtain the concentrations 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 350 and 400 µg/ml. Control petri dish was prepared with 4% v/v DMSO. The final pH of all the media were adjusted to 7.2 to 7.4 before pouring into sterile Petri dishes. Unlike agar plates, agar media having different drug concentrations was placed as a slant in separate test tubes and the fungal inocula (2×10^6 CFU/mL) was streaked and incubated further for at 28°C for 7days. The end points when growth of colonies of control were clearly visible after incubation for 7 days were noted (Mandal et al., 2010). Fluconazole (Fluc) was used as a reference standard. The fluconazole solution was prepared (1 mg/ml) by sterile water. The solution was then added to Czapek-Dox agar media at 45ºC in prepare different

concentrations of fluconazole (5, 10, 25, 50, 100 µg/ml). The final pH of the media was adjusted to 5.4 before preparing slants in sterile test tubes. MIC was recorded for any lowest concentration where complete growth inhibition of microorganisms was observed visually

5.1.2.9. Determination of zone of inhibition (ZOI)

The well-diffusion method was used to determine the ZOI. For that method, NCCLS 2004 guideline was followed. Briefly, agar plates were prepared and 100µL of bacterial/ fungal cell suspension (2×10^6 CFU/mL) was spread on solid agar plates using a sterile spreader. Sterile borer was used to make the wells and the tested compounds at their respective MIC was added to the wells and incubated for 24h at 37°C (for bacteria) and 48-72h at 28°C (for fungus). Fluconazole and amoxicillin were used as a reference standard to compare antifungal and antibacterial activity respectively of the tested compounds. The zone diameters were measured and the experiment was performed in triplicates.

5.1.2.10. Growth kinetic studies

Growth kinetics studies were carried out to those compounds which have shown higher ZOI at their respective MIC as well as for those have comparable and significant MIC values against the MIC of standard drugs for any bacterial and fungal strains used in the experimental design. Thus, selected tested compounds at their concentration of 2× MIC was exposed to gram-positive and gram-negative bacteria to carry out the study. For any given bacterial strain, 1 mL overnight cultured bacterial suspension was taken in two separate test tubes containing 4 mL of fresh nutrient broth media and incubated for 2-3h at 37°C. After that in one tube, the tested compound was added and another tube was used as control (without treatment). At each predetermined time point, 100 µL of bacterial suspension was taken from the tubes, diluted in sterile water to achieve the bacterial concentration of 2×10^6 CFU/mL. From that diluted suspension 100µL was taken to

spread on solid agar plate and incubated for 24h at 37°C. Afterwards, the number of colony was counted for both test and control (Bantar et al., 2008).

For fungal strains, fungal spores at a concentration of 1×10^5 CFU/mL were inoculated 1 mL of Czapek-Dox medium and incubated with or without tested compounds for 48 h with shaking (50 rpm) at 28°C. 100 µL from each medium was taken to dilute in sterile water and spread onto Czapek-Dox agar plate and incubated at 28°C for 48-72h, numbers of colony forming unit were counted for test and control (Kundu et al., 2016).

By calculating the viable counts at each time point, a 24h time- kill curve was plotted by plotting log CFU/mL against time for bacterial strains and for fungal strains 96h time- kill curve was plotted. The experiments were performed in triplicates and expressed as mean ± SD.

5.1.2.11. Scanning electron microscopy (SEM)

Each bacterial and fungal strain was inoculated in two sets, one was treated as a test and another was control. After 2h tested compounds were inoculated in a tube marked as a test. After a sufficient incubation period (for bacteria 37˚C for 24 hrs and for fungus 28˚C for 48-72 hrs), cultures were centrifuged at 3000 rpm for 10min and supernatant was discarded to obtain cells pellet. Cells were resuspended in media specific for bacteria and fungus and kept for 10min and again centrifuged at 3000 rpm for 10min. Fixation of cells was done with 4% glutaraldehyde in 0.1 M Phosphate buffer and kept for 2-3h and again centrifuged at 3000 rpm for 10min and supernatant was discarded. Cells were washed with sterile water and subjected to dehydration by a series of alcohol washing with increasing strength of 30%, 50%, 70%, and 100%, respectively. A drop was put on a clean glass slide and dried. Platinum coating was done and the sample was examined under SEM (Bandyopadhyay et al., 2015).

5.1.2.12. In silico target binding study

AutoDock vina (Trott et al., 2010) using the Lamarckian genetic algorithm (LGA) for the prediction of binding affinity and searching for the optimum binding site together with the AutoDock Tools (ADT) were employed to set up and perform blind docking calculations of the triazole derivatives binding to substrate binding site of respective enzymes. The membrane protein of *Salmonella typhi* (Pdb id: 4KR4); chimeric lanosterol 14α-demethylase (Pdb id: chimeric 1EA1); crystal structure of cytochrome P450 14-alpha-sterol demethylase (Cyp51) from *Mycobacterium tuberculosis* in complex with azole Inhibitors (Podust et al., 2001) were obtained from the Protein Data Bank (www.rcsb.org/pdb) (Rose et al., 2012) at a resolution of 1.60 Å was constructed using Autodock4 package to study the target binding interaction of all the compounds under study. The coordinates of tested compounds were taken from their 2D structures sketched from ACD ChemSketch Freeware and converted to the PDB format using openbabel (O'Boyle et al., 2011).

The target (respective proteins) and the ligand (triazoles) files were prepared using AutoDock Tools. The water molecules were deleted and polar hydrogen atoms and Gasteiger charges were added to both the target molecule and the ligands. All other bonds were made rotatable. The target was saved in pdbqt format and a configuration file was created. Later docking was run. Autodock generated 9 possible binding conformations for each ligand and the root-mean-square (RMS) cluster tolerance was set to 2.0 Å in each run. The binding mode and interactions were analyzed for the significant conformers of the compounds under study. All calculations were performed in a Dell system (3.4 GHz processor, 2GB RAM, 320 GB Hard disk operating system. For each of the docking cases, the lowest energy docked conformation, according to the

Autodock scoring function, was selected as the binding mode. Visualization of the docked pose was done by using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC) molecular graphics program.

Docking was processed with setting of the grid sizes for 4KR4; 30, 26 and 24 and for chimeric 1EA1 40, 34 and 24 along the X, Y and Z axes with 1Å spacing which covered all the active residues. In a similar way the grid center of each target was set for 4KR4; 34.52, 21.86 and 44.96 and for chimeric 1EA1 12.04, 2.87 and 62.50 Å for the protein.

5.1.2.13. Statistical analysis

Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. $P < 0.01$ was considered as statistically significant whereas $P > 0.05$ was considered to be statistically not significant.

5.2. Results and discussion

5.2.1. Determination of minimum inhibitory concentrations (MICs) of the synthesized compounds against bacterial strains

The synthesized compounds (6a-6l) were exposed to 15 different bacterial strains including both gram-negative and gram-positive bacteria and found to be efficacious at a range of MIC values between 25–200 μg/mL. Amoxicillin was used as a reference standard to compare the antibacterial activity of the synthesized compounds. The comparative MIC values of the compounds were tabulated in Table 3. The results showed that majority of the synthesized compounds elicited variable degrees of inhibition against the tested microorganisms. Compounds 6a, 6b, 6c, 6d, 6k, 6l exhibited potent antibacterial activity against *K. pneumonia, B. cereus, P. aeruginosa, B. pumilus, S. typhi, B. subtilis, V. cholerae* at a MIC of 25μg/mL whereas

compound 6e (75 µg/mL) has shown moderate activity compared to the standard having the MIC of 60 µg/mL. Compound 6f (100 µg/mL) has poor efficacy against the same strain where other compounds have shown no activity. Against *Pseudomonas aeruginosa*, compound 6a- 6d, 6k, 6l (MIC of 25-50 µg/mL) have found to be more potent than standard compound (65 µg/mL) where other compounds have found to be no activity. Against the other bacterial strain compounds have shown moderate to less activity compared to the standard like, compound 6e, 6f (50 µg/mL) against *B. polymyxa*; compound 6a, 6k, 6l (50 µg/mL) against *V. cholerae*; compound 6a, 6b, 6e, 6f, 6i (50µg/mL) against *B. bronchiseptica* were moderately active where other compounds have mild bioactivity against the tested bacterial strains, however compared to the standard they have very less to no activity.

5.2.2. Determination of minimum inhibitory concentrations (MICs) of the synthesized compounds against fungal strains

In vitro antifungal activity was evaluated against 7 different fungal strains and the result showed that the synthesized compounds were active against all tested fungi to some extent (Table 3). Among the tested compounds, compound 6a showed potent activity against *Candida albicans* at MIC 15 µg/mL which is nearer to standard fluconazole (12.5 µg/mL), whereas compound 6l (20 µg/mL) was found to be moderately active against *Candida albicans*. Against *Aspergillus niger* compound 6a and 6b (20 µg/mL) were potent whereas compound 6d and 6j (25 µg/mL) were moderately active compared to the standard (18 µg/mL). On the other hand, compound 6b, 6d (20µg/mL) elicited moderate activity against *Candida tropicalis*.

Table 3: MIC values of synthesized compounds (6a-6l)

Organisms	**MIC (µg/mL)**												
Bacterial Strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
S. typhi	25	25	50	50	-	-	-	-	-	-	50	100	10
K. pneumoniae	25	50	25	25	75	100	-	-	-	-	-	-	60
B. Polymyxa	100	100	-	-	50	50	-	-	-	-	100	100	32
B. cereus	50	50	50	25	-	-	-	-	50	75	75	50	12
V. cholerae	50	75	-	-	-	-	75	100	-	-	50	25	25
S. aureus	100	150	100	50	-	-	-	-	-	-	-	-	16
E. faecalis	-	-	-	-	-	-	-	-	200	200	150	100	25
B. bronchiseptica	50	50	-	-	50	50	-	-	50	100	-	-	25
M. luteus	-	-	-	-	-	-	100	150	-	-	25	25	0.5
P. aeruginosa	25	25	50	50	-	-	-	-	-	-	25	25	65

S. sonnei	100	100	-	-	-	-	-	-	-	-	-	-	32
E. coli	-	-	100	100	-	-	100	100	-	-	100	100	10
B. pumilus	25	25	50	25	-	-	-	-	50	50	50	75	2
S. typhimurium	-	-	100	200	200	200	-	-	-	-	150	100	25
B. subtilis	25	25	25	50	-	-	100	100	-	-	100	150	8
Fungal Stains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Fluc**
C. albicans	15	25	50	25	-	-	100	50	50	100	50	20	12.5
C. tropicalis	50	20	100	20	-	-	200	100	-	-	-	-	10
C. neoformans	50	50	-	-	-	-	100	200	200	50	50	50	15
A. niger	20	20	50	25	300	200	100	50	50	25	50	100	18
M. gypseum	-	-	300	200	-	-	500	350	500	300	50	50	16
P. chrysogenum	-	-	300	250	-	-	400	400	-	-	-	-	56

Cladosporium	250	300	-	-	-	-	400	200	250	300	-	-	32

Abbreviation: Amx- Amoxicillin, Fluc- Fluconazole

5.2.3. Determination of zone of inhibition of the tested compounds against bacterial strains

The ZOI of the compounds which showed inhibitory efficacy against individual strains of bacteria and fungus, was calculated at their respective MIC and tabulated in Table 4 like, against *S. typhi* compound 6a, 6b showed the ZOI of 18.5±0.2 mm (at MIC of 25 μg/mL) and 16.5±0.5 mm (at MIC of 25 μg/mL), respectively. Against *K. pneumoniae* compound 6a, 6c, 6d showed ZOI of 16.0±0.4 mm, 9.3±0.3 mm and 13.8±0.1 mm (at MIC of 25 μg/mL), respectively. Compound 6e showed ZOI of 15.4±0.1mm (MIC = 50 μg/mL) against *B. Polymyxa*. Compound 6i and 6l have shown higher ZOI of 15.2±0.2mm and 14.0±0.1 mm, respectively. 6l have a ZOI of 13.6±0.2 mm (MIC = 25 μg/mL) against *V. cholerae*. ZOI of 14.7±0.2 mm (MIC = 50 μg/mL) for compound 6d against *S. aureus*. 11.4±0.4 mm and 9.5±0.2 mm (MIC = 25 μg/mL) for 6k and 6l against *M. luteus*. For compound 6a, 6b, 6k, 6l ZOI was almost 13mm against *P. aeruginosa* at MIC of 25 μg/mL. ZOI of 13.3±0.3 mm and 13.3±0.2 mm were higher for compound 6a against *B. pumilus* and *B. subtilis*, respectively at MIC of 25 μg/mL.

5.2.4. Determination of zone of inhibition of the tested compounds against fungal strains

For fungal strains it was observed that compound 6a have the ZOI of 11.9±0.7 mm (MIC = 15 μg/mL) for *C. albicans*; compound 6b showed 9.2±0.9 mm (MIC = 20μg/mL) for *C. tropicalis* and against *A. niger* compound 6a and 6b have shown almost similar ZOI of 11.2 ±0.6mm and 11.6 ±0.9 mm, respectively at the MIC of 20 μg/mL.

Table 4: ZOI of tested compounds (6a-6l)

Organisms	**ZOI (mm)**												
Bacterial strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
S. typhi	18.5 ±0.2	16.5 ±0.5	15.7 ±0.5	15.1 ±0.2	_	_	_	_	_	_	15.1 ±0.2	11.2 ±0.1	21.0 ±0.9
K. pneumoniae	16.0 ±0.4	11.5 ±0.3	9.3± 0.3	13.8 ±0.1	11.2 ±0.4	10.1 ±0.4	_	_	_	_	_	_	13.5 ±0.9
B. Polymyxa	13.3 ±0.3	16.4 ±0.4	_	_	15.4 ±0.1	12.2 ±0.1	_	_	_	_	11.5 ±0.4	10.6 ±0.2	18.7 ±0.2
B. cereus	14.0 ±0.1	12.1 ±0.2	13.6 ±0.2	15.5 ±0.1	_	_	_	_	15.2 ±0.2	13.5 ±0.4	11.7 ±0.4	13.2 ±0.1	17.3 ±0.2
V. cholerae	11.0 ±0.9	12.0 ±0.1	_	_	_	_	14.1 ±0.2	12.6 ±0.4	_	_	11.4 ±0.4	13.6 ±0.2	13.5 ±0.5
S. aureus	15.3 ±0.2	12.5 ±0.4	12.0 ±0.6	14.7 ±0.2	_	_	_	_	_	_	_	_	16.5 ±0.9
E. faecalis	_	_	_	_	_	_	_	_	11.0 ±0.6	10.2 ±0.2	11.2 ±0.4	11.1 ±0.2	15.2 ±0.4
B.	10.4	10.5	_	_	11.1	10.0	_	_	11.0	12.9	_	_	15.3

bronchisep tica	±0.4	±0.5			±0.2	±0.1			±0.6	±0.2			±0.2
M. luteus	_	_	_	_	_	_	14.2 ±0.1	12.9 ±0.4	_	_	11.4 ±0.4	9.5± 0.2	18.7 ±0.6
P. aeruginosa	14.1 ±0.2	13.6 ±0.2	13.1 ±0.3	11.5 ±0.3	_	_	_	_	_	_	13.5 ±0.4	12.6 ±0.2	16.9 ±0.2
S. sonnei	10.0 ±0.3	9.6± 0.3	_	_	_	_	_	_	_	_	12.0 ±0.3	11.2 ±0.2	15.9 ±0.3
E. coli	_	_	10.8 ±0.3	10.1 ±0.2	_	_	10.7 ±0.3	10.3 ±0.3	_	_	10.7 ±0.1	10.1 ±0.2	17.3 ±0.2
B. pumilus	13.3 ±0.3	11.5 ±0.3	12.0 ±0.3	11.3 ±0.3	_	_	_	_	9.4± 0.2	9.1± 0.4	11.9 ±0.1	11.2 ±0.3	14.4 ±0.1
S. typhimuriu m	_	_	11.0 ±0.1	10.1 ±0.2	12.1 ±0.2	11.1 ±0.3	_	_	_	_	13.7 ±0.2	12.5 ±0.3	16.9 ±0.2
B. subtilis	13.3 ±0.2	11.4 ±0.2	12.1 ±0.3	11.1 ±0.2	_	_	12.4 ±0.2	11.9 ±0.3	_	_	12.8 ±0.2	11.8 ±0.3	13.9 ±0.3
Fungal strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Flu c**
C. albicans	11.9 ±0.7	10.2 ±0.6	10.8 ±0.8	7.8 ±0.7	_	_	7.9 ±0.5	9.6 ±0.8	7.6 ±0.8	11.0 ±0.3	12.2 ±0.3	11.1 ±0.3	16.0 ±0.9

C. tropicalis	9.4 ±0.8	9.2 ±0.9	8.0 ±0.6	9.2 ±0.8	_	_	9.5 ±0.7	9.4 ±1.2	_	_	_	_	16.3 ±0.4
C. neoformans	7.3 ±0.5	8.4 ±0.6	_	_	_	_	8.7 ±0.6	7.5 ±0.7	8.4 ±0.7	10.3 ±0.3	12.9 ±0.3	12.6 ±0.3	14.2 ±0.6
A. niger	11.2 ±0.6	11.6 ±0.9	10.2 ±0.6	9.8 ±0.7	11.3 ±0.9	8.9± 0.7	9.6 ±0.7	11.3 ±0.7	7.43 ±0.6	9.8 ±0.1	14.1 ±0.1	9.9 ±0.1	17.6 ±0.3
M. gypseum	_	_	7.6 ±0.7	6.8 ±0.7	_	_	6.8 ±0.8	8.7 ±0.7	6.8 ±0.7	10.7 ±0.4	11.9 ±0.4	11.1 ±0.3	16.4 ±0.9
P. chrysogenum	_	_	6.6 ±0.8	8.5 ±0.8	_	_	7.7 ±0.6	7.3 ±0.5	_	_	_	_	16.3 ±0.6
Cladosporium	7.5 ±0.6	7.2 ±1.0	_	_	_	_	5.8 ±0.6	8.3 ±0.9	6.5 ±0.8	10.1 ±0.4	_	_	15.2 ±0.9

Amx: Amoxicillin, Fluc: Fluconazole;

Experimental data expressed as mean±SD, n=3

5.2.5. Growth kinetic studies

Compound 6a, 6b, and 6l were most active against the fungal strain *C. albicans*. It was found very interesting that all of the synthesized compounds were active against *A. niger* even compound 6e and 6f which were not effective against the any of the fungal strain (between the tested concentration to determine MIC) used for this experimental design, were active against *A. niger* although the MIC values were high enough i.e. 300 μg/mL, 200 μg/mL, respectively,

compared to the standard drug fluconazole (16 μg/mL). However, compound 6b and 6a showed potent antifungal activity against *A. niger* with ZOI of 11.6 ±0.9 mm and 11.2 ±0.6 mm at the MIC of 20 μg/mL.

5.2.6. Scanning electron microscopy

SEM was carried out to assess morphological changes in bacterial cells following the exposure of most active compounds against selective bacterial as well as fungal strains; like, for bacterial strains, *B. pumilus* was treated with compound 6a and *P. aeruginosa* was treated with compound 6b whereas for fungal strains, *C. tropicalis* was treated with compound 6b and *C. albicans* was treated with compound 6a. The SEM analysis showed clear morphological changes of bacterial cells when treated with tested compounds. Fig 33K and 33L depicts the SEM image of control and treated *B. pumilus*, respectively. SEM micrographs of *B. pumilus* revealed that when the bacterium was exposed to the tested compound the cells disrupted following alterations in the cell integrity. *P. aeruginosa* when treated with the tested compound (Fig 33N), appeared to swell with loss of its normal morphology as depicted by the control (Fig 33M). Moreover, based on the pathogenic mechanisms of *B. pumilus* and *P. aeruginosa*, an alternative strategy for the treatment of the infections caused by these organisms is of supreme importance in current scenario.

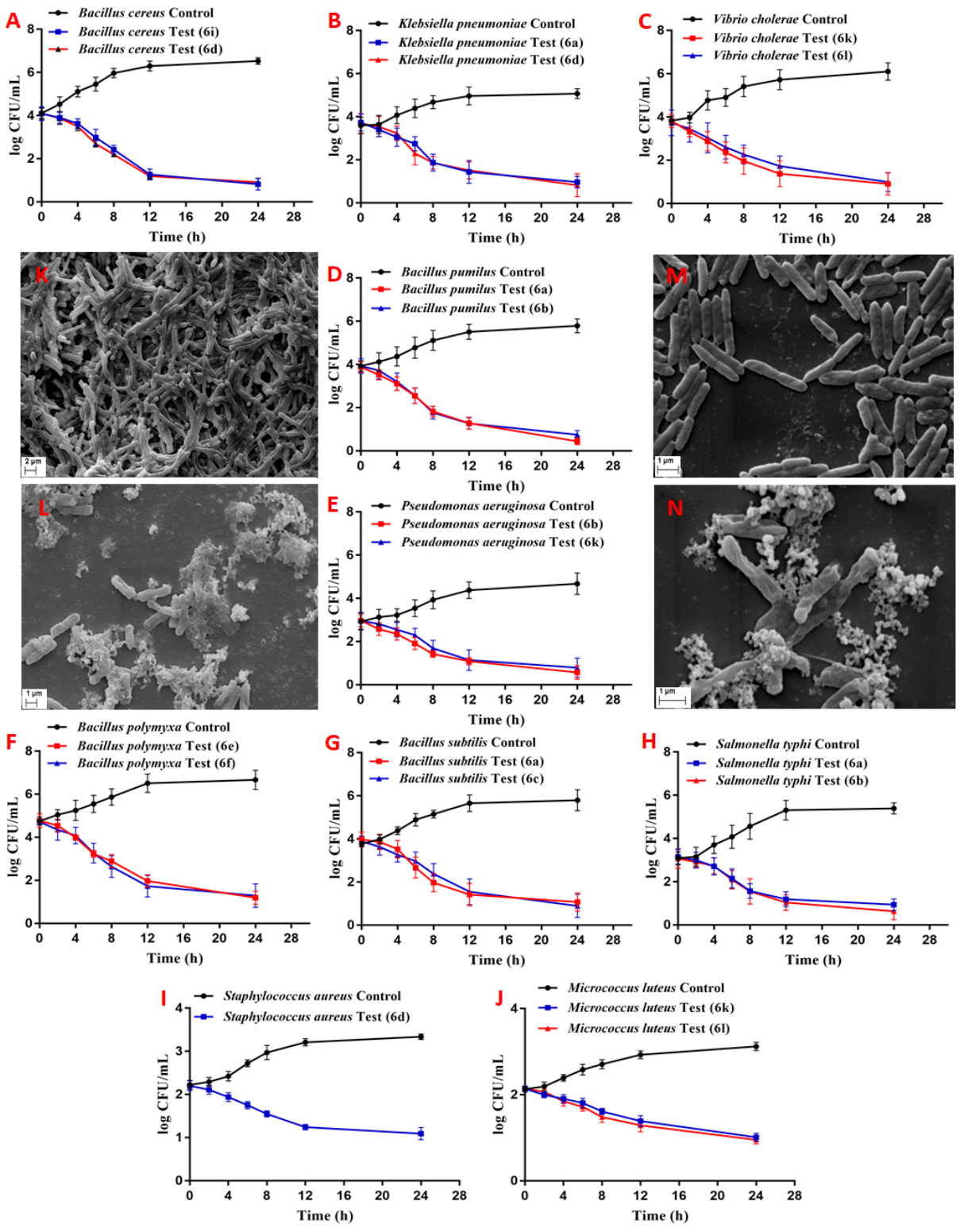

A
Bacillus cereus Control
Bacillus cereus Test (6i)
Bacillus cereus Test (6d)
log CFU/mL
Time (h)
B
Klebsiella pneumoniae Control
Klebsiella pneumoniae Test (6a)
Klebsiella pneumoniae Test (6d)
log CFU/mL
Time (h)
C
Vibrio cholerae Control
Vibrio cholerae Test (6k)
Vibrio cholerae Test (6l)
log CFU/mL
Time (h)
K
2 µm
D
Bacillus pumilus Control
Bacillus pumilus Test (6a)
Bacillus pumilus Test (6b)
log CFU/mL
Time (h)
M
1 µm
L
1 µm
E
Pseudomonas aeruginosa Control
Pseudomonas aeruginosa Test (6b)
Pseudomonas aeruginosa Test (6k)
log CFU/mL
Time (h)
N
1 µm
F
Bacillus polymyxa Control
Bacillus polymyxa Test (6e)
Bacillus polymyxa Test (6f)
log CFU/mL
Time (h)
G
Bacillus subtilis Control
Bacillus subtilis Test (6a)
Bacillus subtilis Test (6c)
log CFU/mL
Time (h)
H
Salmonella typhi Control
Salmonella typhi Test (6a)
Salmonella typhi Test (6b)
log CFU/mL
Time (h)
I
Staphylococcus aureus Control
Staphylococcus aureus Test (6d)
log CFU/mL
Time (h)
J
Micrococcus luteus Control
Micrococcus luteus Test (6k)
Micrococcus luteus Test (6l)
log CFU/mL
Time (h)

Fig 33. Time dependent *in vitro* growth curve of *Bacillus cereus* (A), *Klebsiella pneumoniae* (B), *Vibrio cholerae* (C), *Bacillus pumilus* (D), *Pseudomonas aeruginosa* (E), *Bacillus polymyxa* (F), *Bacillus subtilis* (G), *Salmonella typhi* (H), *Staphylococcus aureus* (I), *Micrococcus luteus* (J) at their 2×MIC values against test compounds and Scanning electron micrograph of *Bacillus pumilus* (K) before treatment, (L) after treatment; *Pseudomonas aeruginosa* (M) before treatment, (N) after treatment; Experimental data expressed as mean±SD, n=3; $P < 0.01$ was considered as statistically significant

Fig 34A and 34B are the SEM images of control and treated *C. albicans*, respectively, whereas, Fig 34E and 34F shows the SEM images of control and treated *C. tropicalis*, respectively. In both the cases structural changes in the SEM studies appeared progressively due to cohesion, diffusibility, agglomeration, and ultimate structure distortion and disintegration.

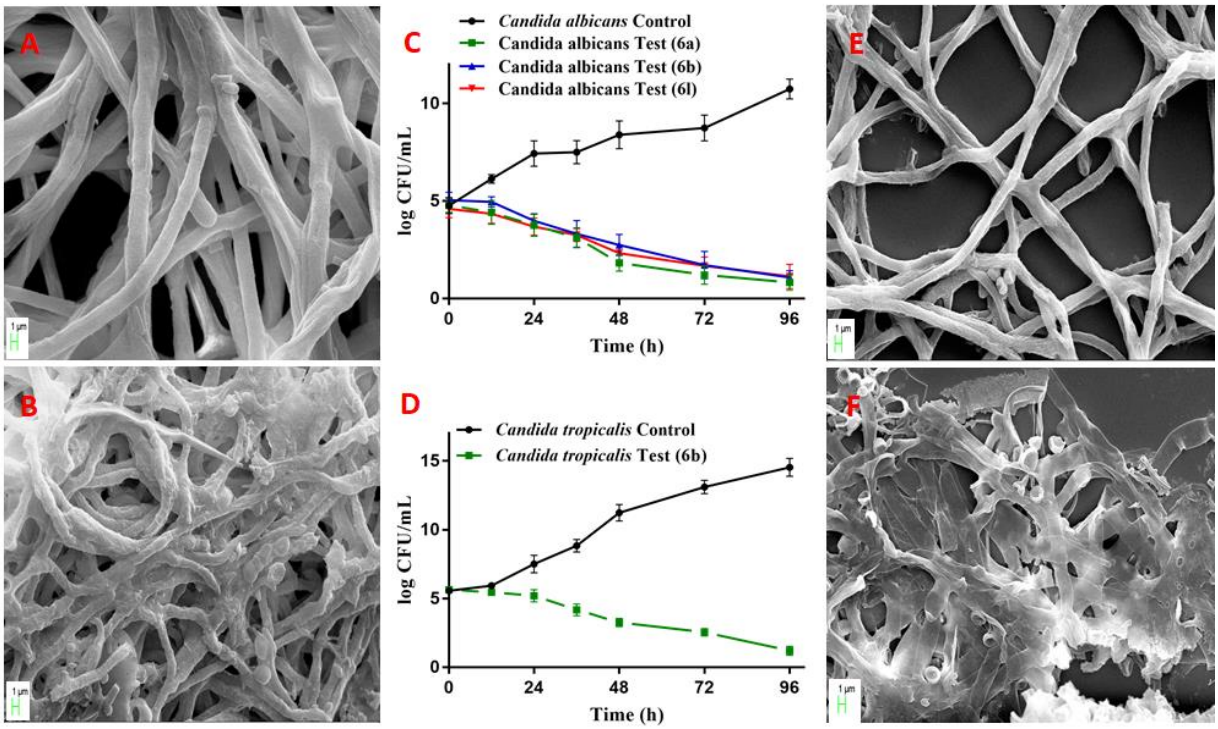


Fig 34. Scanning electron micrograph of *Candida tropicalis* (A) before treatment, (B) after treatment; *Candida albicans* (E) before treatment, (F) after treatment and Time dependent in

vitro growth curve of Candida albicans (C) and Candida tropicalis (D) at their 2×MIC values against test compounds; Experimental data expressed as mean±SD, n=3, $P < 0.01$ was considered as statistically significant

Growth kinetic study was done against the bacterial strains like *B. cereus*, *K pneumoniae*, *V. cholerae*, *B. pumilus*, *P. aeruginosa*, *B. polymyxa*, *B. subtilis*, *S. typhi*, *S. aureus* and *M. luteus* for the tested compounds which showed higher ZOI at their respective MIC (Fig 33A- 33J; respectively). Whereas, for fugal strains, compound 6a, 6b and 6l were studied for the growth kinetics evaluation against *C. albicans* and 6b for *C. tropicalis* displayed in Fig 34C and 34D; respectively. Although, almost all synthesized compounds have shown antifungal activity against *A. niger*, the growth kinetic study couldn't be performed as the *A. niger* strains forming very dark brown spores which couldn't be counted visually. From the growth kinetics data of bacteria as well as fungus from Fig 33 and 34, respectively, it could be observed that the all best active compounds showed bacteriostatic and fungistatic activity.

It could be observed from the antimicrobial data that synthesized aryl substituted 1,2,4- triazole linked hydroxamic acid derivatives showed moderate to high activity against tested bacterial as well as fungal strains. Ji et al have shown that lanosterol-14α-demethylase is one of the primary targets of antifungal drug discovery (Hase et al., 1971) like, by targeting ergosterol biosynthetic pathway through inhibiting that key enzyme, lanosterol 14-alpha demethylase, azoles exert their antifungal activity. The molecular mechanism of this inhibition occurred by the binding of free nitrogen atom present in azole ring with the iron atom present in the enzyme. A toxic compound formed through the metabolism of 14-alpha methylated sterol, which couldn't replace ergosterol (Rossello et al., 2002). Among the two main distinct types of azoles, triazoles are effectively used against invasive fungal infections (D'Enfert et al., 2014). The molecular mechanism of

triazole as an antifungal has already been established. All the compounds considered for the study possessed 1,2,4-triazole ring in it, therefore all the compounds exhibited affinity towards the enzyme. The intensity of binding was high in compounds 6a, 6b and 6l, which may be due to the ring nitrogen and the electronegative nitro group present in aryl group attached to the central triazole respectively. Our titled compounds resemblance with a popular histone deacetylase inhibitor namely suberoylanilide hydroxamic acid (SAHA) and it was already reported by Bauer group that histone deacetylase I could be a potential antifungal target (Bauer et al., 2016), which in turn support the antifungal activity of our synthesized compounds. Triazole is well known for its antimicrobial activity. However, electron-rich aryl substitutions at 5- position of the 1,2,4-triazole could be an added benefit along with the linked hydroxamic acid. For many decades, the antimicrobial activity of different hydroxamic acid derivatives was evaluated. It was reported that hydroxamic acid is a potent as well as a specific inhibitor of bacterial urease (Hase et al., 1971). So, the antimicrobial activity of the synthesized compounds might be due to the partial effect of hindered urea metabolism by the hydroxamic acid part. It was also reported that presence of -CONHOH group is very much necessary for the inhibition of microbial urease. In addition, alkyl group present in the hydroxamic acid part of the synthesized compounds influenced the effectiveness.

5.2.7. In silico target binding study

The binding mode of the highest active test compound exhibiting appreciable antimicrobial activity was investigated by docking studies. Crystal structure of *Salmonella typhi* was obtained from protein repository. On the other hand, no experimental structural information on the active site of the target enzyme *Candida* P450DM is available. It is observed from an extensive literature search that high homology exists between the mycobacterium P450DM and Candida

P450DM. Following the method of Rossello et al (Rossello et al., 2002) the chimeric enzyme for the *Candida albicans* (CACYP51) was developed from that of mycobacterium P450DM (MT CYP51) extracted from the protein data bank (PDB entry code 1EA1). The residues that were arranged in a range of 7Å from fluconazole were substituted with those of Candida P450DM. Substitutions were made by replacement of the residues Pro77, Phe78, Met79, Arg96, Met99, Leu100, Phe255, Ala256, His258, Ile322, Ile323 and Leu324 by Lys77, His78, Leu79, Leu96, Lys99, Phe100, Met255, Gly256, Gln258, His322, Ser323 and Ile324, which were thought to be necessary for the ligand-receptor interaction. Compound 6k, 2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide was within the active site of 4kr4. The active site composition was procured from research collaboratory for structural bioinformatics (rcsb) and the binding pattern of ampicillin in Figure 35B was set as standard. Almost all the docked conformers occupied the proximal vicinity of the active site which is clearly understood from Figure 35A.

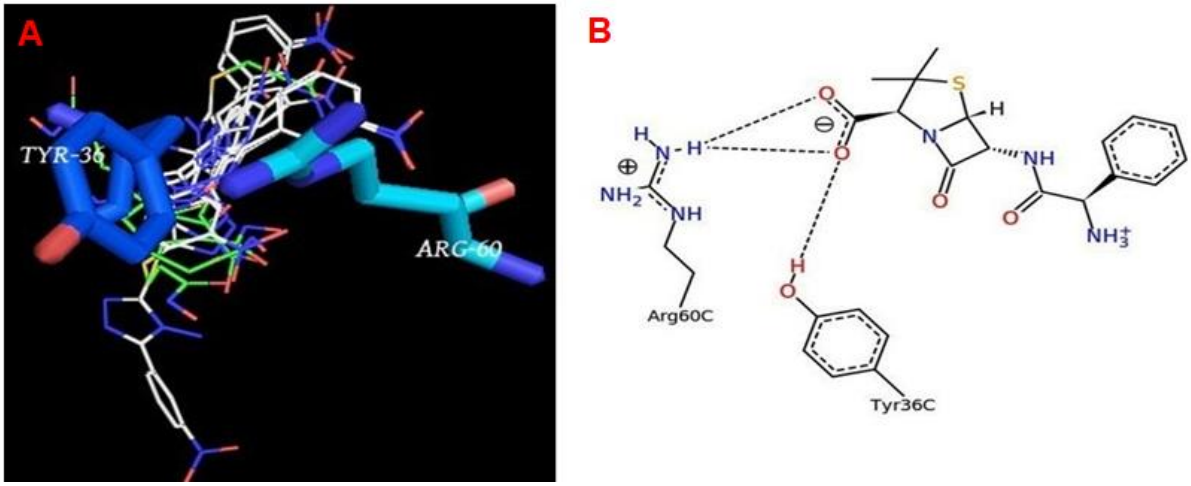


Fig. 35. A; Cluster of conformers within the active site of the target protein of *Salmonella typhi* (pdb id: 4kr4), B; Poseview of standard ampicillin within the active site of 4kr4

Based on the fact that azoles exhibit antifungal activity by inhibition of a cytochrome P450 enzyme, lanosterol-14-α-demethylase during the biosynthesis of ergosterol leading to accumulation of 14-α-sterols in the fungal cell membrane causing destabilization and membrane degradation, it was thought worthwhile to investigate the binding mode of the highest active test compound, 6c, 2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide in the active site of chimeric enzyme of cytochrome P450-14-α-sterol demethylase from *Candida albicans* (Candida P450DM) (chimeric 1EA1).

Binding mode analysis of test compound 6c in the active site of chimeric 1EA1 (Figure 36A) showed that the test compound was very close to the porphyrin ring, HEM 470, similar as that of the co-crystallized ligand fluconazole. Docking poses of the test compound suggest the presence of active site residues leu321, tyr76 as in case of fluconazole (Figure 36B).

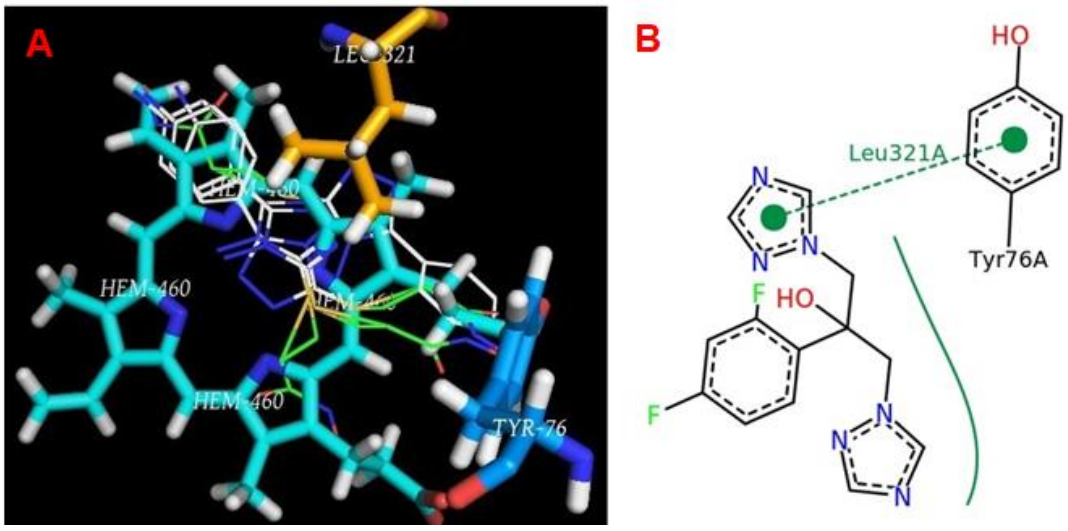


Fig 36. A; Cluster of conformers within the active site of the target enzyme of *Candida albicans* (pdb id: chimeric 1ea1), B; Poseview of standard fluconazole within the active site of 4kr4

5.3. Conclusion

A series of twelve 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives (6a-6l) were synthesized and evaluated for their *in vitro* antimicrobial potency and found to be potent to moderately active in inhibiting the pathogenic growth. The significant antimicrobial property may be attributed to the putative substructure of triazole (which itself can form ionic interaction with a various microbial target; the most acceptable is lanosterol-14α-demethylase in *Candida albicans*) and the salient feature of the hydroxamic group. The aryl group with its substituent leaves a mixed opinion with respect to the biological interaction. The electron donating ability of methyl, mercapto, amino group strengthen the binding affinity of the aryl group with the active site of the target might be one of the strong supporting evidence for their potency, but in the same ground how this nitro group being a strong electron group helps in lifting the chemotherapeutic index is difficult to establish. Therefore, the series requires to be well extended. Thus, it could be concluded that the synthesized 1,2,4-triazole- linked hydroxamic acid derivatives with electron donating groups at 5-position of the 1,2,4-triazole moiety may provide a therapeutically effective chemical framework from which potential antimicrobial agents may be developed further. Therefore, further optimizations of this prototypical molecular framework with some diversified molecular fragments may generate new drug entities having potent antimicrobial activity.

Chapter 6

6. Antioxidant study

Oxidation processes are intrinsic to the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms (Ślusarczyk et al., 2009). Free radicals are molecules, ions or atoms with unpaired electrons in their outermost shell of electrons (Dakubo et al., 2010). These species, which are constantly formed in the human body, can become toxic when generated in excess or in the presence of a deficiency in the naturally occurring antioxidant defenses. High levels of free radicals can cause damage to biomolecules such as lipids, proteins, enzymes and DNA in cells and tissues. This may result in many diseases such as cancer, diabetes, cardiovascular and autoimmune diseases, and neurodegenerative disorders, aging, and other diseases through the violent reactivity of the free radicals (Torreggiani et al., 2005; Karalı et al., and Patil et al., 2013). Antioxidants are important compounds that reduce or neutralize free radicals, thus protecting the cells from oxidative injury (Azam et al., 2010). Heterocyclic compounds have been used to be the most researched chemical entity in the area of organic chemistry, having wide verities of application in the field of pharmaceuticals to agro-industries (Maddila et al., 2013). Among the heterocyclic compounds, nitrogen-containing heterocycles like triazole grab the attention of the scientific community over the decades. 1,2,4-Triazoles is an interesting isomer of triazoles having a diversified pharmacological activities such as anticancer, antidepressant (Bhatnagar et al., 2011), antibacterial (Karabasanagouda et al., 2007), antifungal (Sztanke et al., 2008), anti-inflammatory, antileishmanial (Süleymanoğlu et al., 2017) and antiviral activities (Küçükgüzel et al., 2008) etc. It could be found from different experimental design associated with the 1,2,4-triazole that it also possesses superior antioxidant activity. Cetin et al demonstrated that phenol and pyridine substituted 1,2,4-triazole attributed exceptional antioxidant properties (Cetin et al.,

2015). Pokuri et al have designed and synthesized different derivatives of 1,2,4 triazoles and found significant antioxidant activity over the standard ascorbic acid (Pokuri et al., 2014). Karrouchi et al have synthesized a series of pyrazole containing derivatives of 1,2,4- triazoles and found a significant antioxidant activity for all of the synthesized compounds (Karrouchi et al., 2016).

Hydroxamic acids (R-CO-NH-OH) are also an important class of chemical compounds that could be derived from hydroxylamine (NH_2OH) and carboxylic acids (R-COOH). This class of compounds has the ability to chelate metal ions and known to have multiple biological activities (Bernardo et al., 2017). They found to have potent antioxidant activities. Like, Marijana et al have synthesized hydroxamic acid derivatives of nonsteroidal anti-inflammatory drugs (NSAIDs) and found significant radical scavenging, antioxidant and metal chelating activities. They have found that N-methylhydroxamic acid of diclofenac showed potent radical scavenging activities (Končič et al., 2009). Bernardo et al have synthesized a series of hydroxamic acid derivatives with different chemical groups like aliphatic, aromatic and amino acids and found a high antioxidant activity of almost all of them.

6.1. Materials and Methods

6.1.1. Materials

All the chemicals were procured from Sigma-Aldrich, India, Spectrochem, Merck India Pvt. Ltd. Gallic acid, ascorbic acid (AA), DPPH (1,1- diphenyl-2-picrylhydrazyl) and TPTZ (2,4,6 tripyridyl- S-triazine) were purchased from Sigma-Aldrich, India. Folin–Ciocalteu reagent, TBA (thiobarbituric acid), 2- deoxy-D-ribose and NBT (nitro blue tetrazolium) were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate, phosphoric acid, acetic acid, HCl (hydrochloric acid), sulfuric acid, EDTA (ethylenediaminetetraacetic acid), sodium acetate

trihydrate, $FeCl_3 \cdot 6H_2O$ (ferric chloride), $FeSO_4 \cdot 7H_2O$ (ferrous sulphate), H_2O_2 (hydrogen peroxide), sodium hydroxide, TCA (trichloroacetic acid), hydroxylamine hydrochloride, sodium phosphate, ammonium molybdate, sodium nitroprusside, NED (N-(1-naphthyl)-ethylenediamine dihydrochloride), sodium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, starch, sodium potassium tartrate, DNS (3, 5- dinitrosalicylic acid), dimethyl sulfoxide (DMSO) and methanol were procured from Merck, India. The water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for all tests. All other reagents used were of analytical grade.

6.1.2. Methods

6.1.2.1. DPPH radical scavenging activities

Antioxidant activity of the synthesized compounds (6a-6l) was evaluated by determining its scavenging ability against DPPH radical (Jing et al., 2017). DPPH was dissolved in ethanol and 100µl (0.1mM) of that solution was added in a microtiter plate. 50µl of different concentrations (2.5–400µg/ml) of the tested compounds were added further. Instead of tested compounds, Milli-Q water was used to prepare the control. Then the microtiter plate was incubated in dark for 30 min at room temperature. The absorbance was measured at 517nm using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA). To evaluate the contribution of the sample color itself, samples without DPPH were prepared and absorbance was measured similarly. Ascorbic acid was taken as standard. The free radical scavenging activity was determined by the following equation,

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100\%$$

Where A_c denoted the absorbance of the control and A_s denoted the absorbance of the sample. IC_{50} value of the synthesized compounds was determined. IC_{50} value is the half maximal

inhibitory concentration to measure the potency of a substance in inhibiting 50% absorbance of initial DPPH concentration.

6.1.2.2. Total antioxidant capacity

The total antioxidant capacity (TAC) of the synthesized compounds (6a-6l) was evaluated by the phosphomolybdenum method (Hammi et al., 2015) with some modifications. This method relies on the fact that if a sample having antioxidant property then it could reduce Mo(VI) to form Mo(V). In acidic pH, the green phosphate/Mo(V) complex was formed and the intensity of which could be measured at 695nm. Briefly, 20µl of the tested compounds of varying concentrations (2.5- 320µg/ml) was mixed with 200µl of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The mixture was incubated for 90 min at 95°C and cooled down to room temperature. The absorbance was measured at 695nm against a blank. The antioxidant activity was calculated from the standard curve of the gallic acid and expressed as mg GAE/g.

6.1.2.3. ABTS [2,2´-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical cation scavenging activities

ABTS radical cation ($ABTS^{+}$) scavenging activity was assayed according to the method of (Re et al., 1999), with a slight deviation. ABTS solution (7mM) was reacted with potassium persulfate (2.45mM) to produce $ABTS^{+}$ solution and kept in the dark for at least 16 h at room temperature. The solution was then diluted with PBS (pH 7.4) to get an absorbance of ~0.750 at 734nm. 1ml of diluted $ABTS^{+}$ was mixed with 10µl of the synthesized compounds at different concentrations (1.56- 50µg/ml) and allowed to react at 30˚C for 20 min. The sample blank was prepared by $ABTS^{+}$ solution and ethanol. The percentage inhibition of absorbance was measured at 734nm.

Ascorbic acid was used as a reference standard. The radical scavenging activity of the samples was calculated by the following equation

$$\% \text{ Inhibition} = \frac{\text{Abs}_c - \text{Abs}_s}{\text{Abs}_c} \times 100\%$$

Where Abs_c was the absorbance of the control reaction mixture and Abs_s was the absorbance of the sample reaction mixture. IC_{50} value of the synthesized compounds was determined and compared with ascorbic acid using the formula.

6.2. Results and discussion

6.2.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activities

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is one of the most rapid and simple technique to determine the antioxidant efficacy of tested compounds (Barbuceanu et al., 2014). DPPH• is a stable free radical which is commercially available. The theory behind the assay is that DPPH• accept hydrogen atom to pair with a free odd electron from any tested compound having hydrogen donating capability and thus the tested compound is said to be an antioxidant. In this study, compound 6a-6l was evaluated for their free radical scavenging activity in the presence of DPPH where ascorbic acid was used as standard antioxidant. The absorption of purple color DPPH could be measured by UV spectrophotometer at a wavelength of 517 nm. However, in the presence of an antioxidant, the color turns into yellow. The reduction of color intensity reflects the ability of an antioxidant as a radical scavenger (Zhou et al., 2013).

The IC_{50} values were measured for tested compounds and represented in table 5. Here the IC_{50} value denoted the effective concentration at which 50% of the initial DPPH was scavenged. The radical scavenging activity of the tested compounds was compared with the positive control, ascorbic acid. It was found from table 5 that compound 6a-d, 6k and 6l have potent antioxidant activity with IC_{50} values of 10.79 ± 3.76, 5.71 ± 2.29, 8.46 ± 2.96, 7.52 ± 1.55, 8.07 ± 2.34, 5.02

± 1.68µg/ml, respectively, compared to the standard ascorbic acid having the IC_{50} of 15.15± 1.72µg/ml. The superior antioxidant activity demonstrated that pyridyl, *p*-amino benzyl and *m*-nitro benzyl substitutions at R^1 position was important. As they provide a resonance effect in the structure of the tested compounds, making them more reactive which could be justified by the work done by Cetin et al, who reported that pyridine substituted 1,2,4-triazole compounds have a better prospect to be an antioxidant [**7**].

6.2.2. ABTS [2,2´-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical cation scavenging activities

It was another method to determine the antioxidant activity of tested compounds. Except for compound 6e and 6f, all others have shown superior to moderate antioxidant activities compared to the standard, ascorbic acid (55.84± 0.23µg/ml). Compounds 6b showed excellent ABTS radical cation scavenging activity with IC_{50} value of 4.12 ± 0.5µg/ml and compounds 6a, 6c, 6d, 6k and 6l were also active as they nearly fully scavenged ($ABTS^+$) with IC_{50} value of 14.65 ± 0.55, 23.53 ± 3.62, 16.41 ± 1.02, 21.82 ± 0.69 and 18.54 ± 3.92µg/ml, respectively. It was observed that the reaction was very fast and completed within 1 min. During the reaction time, the changes in absorbance were very negligible. It was also found that like DPPH assay results, ABTS assay has also shown that, compound 6a-d, 6k and 6l have superior radical scavenging activities which could be due to the similar effect described in case of DPPH assay.

6.2.3. Total antioxidant capacity

A green phosphate/Mo (V) complex was produced in the phosphomolybdenum method and the absorption was measured at 695 nm. The absorbance value was proportional to the antioxidant potency of the tested compounds. The reducing capacity or electron donating potency of the antioxidants to molybdenum was evaluated in the assay. The reducing ability of a chemical

entity may play as a remarkable indicator of its potential antioxidant activity. From Table 5 it could be found that compounds 6a, 6b, 6d, 6e, 6f and 6k have shown greater total antioxidant capacity compared to the other tested compounds. Among all of the compounds 6a **(**637.5 ± 4.42**),** 6b **(**233.59 ± 1.10**)** and 6e **(**348.44 ± 4.42**)** showed the highest activity.

Table 5 DPPH radical scavenging, ABTS and total antioxidant inhibitory activities of the tested compounds.

Tested Compounds	IC_{50} (µg/ml)		Total antioxidant capacity (mg GAE/g of the tested compounds)
	DPPH	ABTS	
6a	10.79 ± 3.76	14.65 ± 0.55	637.5 ± 4.42
6b	5.71 ± 2.29	4.12 ± 0.50	233.59 ± 1.10
6c	8.46 ± 2.96	23.53 ± 3.62	61.72 ± 3.31
6d	7.52 ± 1.55	16.41 ± 1.02	124.21 ± 5.52
6e	99.64 ± 0.82	84.46 ± 1.96	348.44 ± 4.42
6f	118.22 ± 0.83	71.38 ± 3.07	172.66 ± 1.10
6g	75.58 ± 3.21	49.40 ± 0.68	13.28 ± 1.11
6h	68.29 ± 4.21	45.30 ± 2.65	20.31 ± 4.41
6i	69.96 ± 3.36	41.57 ± 2.91	62.5 ± 2.21
6j	47.85 ± 3.49	33.74 ± 2.02	76.56 ± 4.42
6k	8.07 ± 2.34	21.82 ± 0.69	148.44 ± 4.42
6l	5.02 ± 1.68	18.54 ± 3.92	61.72 ± 3.31
Ascorbic acid	15.15± 1.72	55.84± 0.23	-

Data expressed as Mean ± SD, n=3

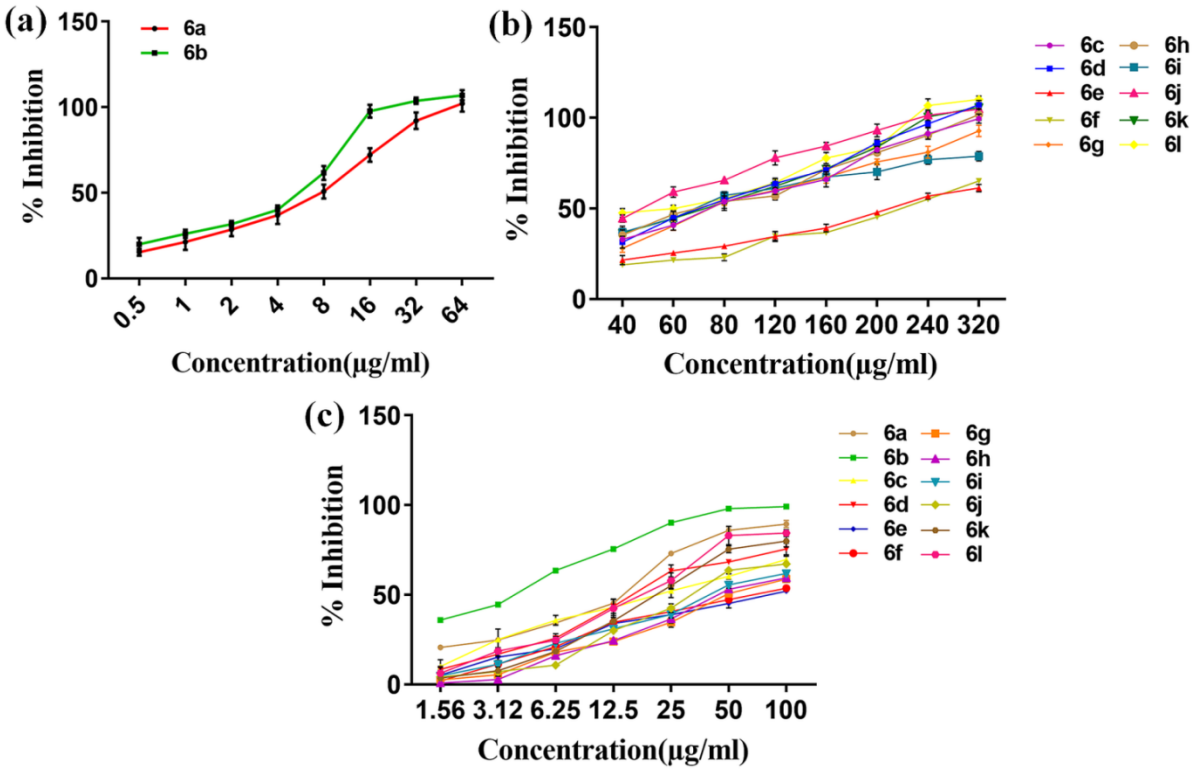


Fig 37. Free radical scavenging activity (a & b DPPH radical; c, ABTS radical) of the synthesized compounds 6(a-l)

6.3. Conclusion

It could be concluded that compound 6b has shown potent antioxidant activity among all other tested compounds. Though other tested compounds have shown moderate to low antioxidant activity compared to the standard, ascorbic acid. It could be suggested from the fact that pyridyl substitution at R^1 position was important to exert superior antioxidant properties.

Chapter 7

7. Anticancer study

Breast cancer is the leading cause of cancer death in women worldwide (Jemal et al., 2011). One of the reasons for such a high mortality is invasive behavior of breast cancer cells. Therefore, breast cancer often progresses from the nonmetastatic and therapy-responsive phenotype to the highly invasive and metastatic phenotype, which is usually resistant to standard therapeutic procedures (Pasqualini et al., 2004 and Thompson et al., 2002). Cancer metastasis consist from several interdependent processes including uncontrolled growth of cancer cells, their invasion through surrounding tissues, migration to the distant sites of the human body, and adhesion, invasion and colonization of other organs and tissues (Price et al., 1997). In addition, tumor growth and metastasis also require angiogenesis, the formation of blood vessels by capillaries sprouting from pre-existing vessels (Folkman et al., 1995). Therefore, suppression of growth and invasiveness of cancer cells, and cancer cells mediated angiogenesis could lead to the inhibition of cancer metastasis and would eventually further increase survival of breast cancer patients. Besides the antioxidant potential of hydroxamic acid derivatives, antitumor or anticancer activity is also well established. Suberoylanilide hydroxamic acid (SAHA) is a well known example of hydroxamic acid derivatives. SAHA is a potent histone deacetylase inhibitor and clinically approved to treat cutaneous T-cell lymphoma. However, the anticancer efficacy and apoptosis effect of SAHA was evaluated against different types of cancer (Munster et al., 2009 and Garcia-Manero et al., 2008). Zhao et al reported the SAHA as potent antitumor agent for large-cell lung carcinoma. It has been found from the work that SAHA could inhibit the cell proliferation by arresting the cell cycle G2/M phase in a dose- and time-dependent manner. They also found potent tumor suppression in nude mouse xenograft model (Zhao et al., 2014). According to Peela et al, against the treatment of solid tumors SAHA have provided conflicting results even in

clinical trials. However, they have found significant inhibition of cellular migration as well as decreased microtubule polarization in case of SAHA against breast cancer cell line, SUM159 (Peela et al., 2017). Besides SAHA, there were several derivatives of hydroxamic acids have shown effective anticancer efficacy. Like, Tardibono et al have synthesized several hydroxamic acid containing 1,4-Benzodiazepines and found to have good anticancer activity (Tardibono et al., 2009). A series of diaryl ether hydroxamic acids were synthesized by Zhu et al and some of them have shown inhibitory activity against four cancer cell lines (Zhu et al., 2012).

The number of research work to develop superior antioxidant as well as anticancer agents implies the necessity to treat free radical associated diseases to life threatening cancer. Considering the above fact and in a search for new antioxidant and anticancer agent, we have evaluated the antioxidant and anticancer activity of a series of 4-amino-5-((aryl substituted)-4h-1,2,4-triazole-3-yl)thio linked hydroxamic acid derivatives, which have been synthesized and reported by us recently. In this experimental design we have evaluated the antioxidant potency by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, [2,2´-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] (ABTS) radical cation scavenging activities as well as total antioxidant capacity. *In vitro* anticancer activity was evaluated against different breast cancer cell line like MDA-MB-231, MCF7 as well as against HCT116, cell line of colorectal carcinoma. In vivo antitumor efficacy was evaluated against EAC induced mice model.

7.1. Materials and Methods

7.1.1. Materials

All the chemicals were procured from Sigma-Aldrich, India, Spectrochem, Merck India Pvt. Ltd., and the media were of purchased from Himedia Laboratories. DMEM media, FCS, MTT reagent was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. HCl (hydrochloric

acid), sulfuric acid, EDTA (ethylenediaminetetraacetic acid), dichloromethane (DCM), dimethyl sulfoxide (DMSO) and methanol were procured from Merck, India. The water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for all tests. All other reagents used were of analytical grade.

7.1.2. Methods

7.1.2.1. *In vitro* anticancer activity

MTT assay was employed to carry out *in vitro* anticancer activity. All of the synthesized compounds were tested against HCT 116, MDA-MB-231 and MCF-7 cell lines. Cells were cultured in 10% FCS supplemented DMEM medium and maintained in a 5% CO_2 incubator at 37˚C. Briefly, 100µl of supplemented media containing 5×10^3 cells were seeded into the predetermined wells of 96 well plates and kept into 5% CO_2 incubator for 24h at 37˚C. After that media was replaced carefully with 100µl of fresh media containing different predetermined concentrations of tested compounds and kept for another 24h at previous incubation condition followed by careful removal of media and 10µl of MTT reagent (5mg/ml) was added and incubated in dark for 4h. 100µl of DMSO was added to dissolve the formazan crystals after removing the supernatant from each well and followed by the mechanical shaking for 10-15 min. Absorbance was taken at 570nm. Percentage inhibition was calculated by the following equation,

$$\%\ \text{Inhibition} = \frac{A_{control} - A_{test}}{A_{control}} \times 100\%$$

Where, $A_{control}$ and A_{test} were the absorbance of control and test, respectively.

7.1.2.2. Study of *in vivo* anticancer activity

7.1.2.2.1. Experimental animals

Female Swiss albino mice (20-25g body weight) were used to carry out the *in vivo* experiments. The animals were kept at standard laboratory conditions (Temp. 25 ± 2ºC and humidity 55 ±

10%) under alternating light and dark environment (12h/12h). Animals were fed with standard food pellet diet and drinking water was provided ad libitum. The guidelines of Institutional Animal Ethics committee ((Ref. No. AEC/PHARM/1401/2014), India was followed to carry out all experimental procedures. The animals were kept for one week in laboratory condition before initiation of these *in vivo* experiments.

7.1.2.2.2. Median lethal dose (LD_{50}) determination

The acute toxicity study was done by Reed et al (Reed et al., 1938) with some necessary modifications. Three synthesized compounds (6b, 6j, 6l) were tested by this experimental design. Each tested compounds were administered orally (single dose) to five groups of mice (six mice in each) at different doses (1000, 1500, 2000, 2500 and 3000mg/kg). Control groups received only normal saline orally under overnight fasting condition. The sign of toxicity and mortality were recorded within 24-72 h for all groups of animals. It was found that the LD_{50} value was almost 2500 mg/kg body weight for all of the tested compounds.

7.1.2.2.3. *In vivo* tumor cell transplantation

The Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. To evaluate the anticancer effect of the tested compounds (6b, 6j, 6l) *in vivo*, EAC cell lines were used because according to the Sahyon et al, EAC cells induced mammary gland carcinoma in female mice mimic the breast cancer model in human (Sahyon et al., 2018). The ascetic fluids were collected from peritoneal cavity by sterile syringe and kept in a sterile petri-plates followed by repeated washing with PBS to remove the non-adherent cells. Then the ascetic fluids were centrifuged and the pellets were suspended in sterile isotonic saline. Cell viability was checked by trypan blue exclusion assay and viability was

assessed to be 95% and these viable cells were used to carry out the further experiments (Chattopadhyay et al., 2002 and Bhattacharyya et al., 2003).

7.1.2.2.4. Experimental design

The animals were divided into twelve groups containing six animals (n=6) in each group. All the experimental animals of each group were injected with EAC cells (2×10^6 cells/ mouse) through intraperitoneal route, except 'Group I' and the day of cell inoculation was treated as 'day 0' on which no treatment was given. The group distributions were as follows

Group I: was denoted as normal control (normal saline, 5 ml/kg body weight, i.p). Animals were injected with sterile distilled water for injection.

Group-II: was denoted by EAC control. Animals were treated with EAC cells by intraperitoneal injections from day 1 to day 10.

Group III: was designed as reference group. Animals were treated with standard drug [5-Fluoro Uracil (5-FU) 20mg/kg body weight] by intraperitoneal injections from day 1 to day 10.

Group IV: Animals were treated with tested compound, 6b (25mg/kg body weight) from day 1 to day 10.

Group V: Animals were treated with tested compound, 6j (25mg/kg body weight) from day 1 to day 10.

Group VI: Animals were treated with tested compound, 6l (25mg/kg body weight) from day 1 to day 10.

Group VII: Animals were treated with tested compound, 6b (50mg/kg body weight) from day 1 to day 10.

Group VIII: Animals were treated with tested compound, 6j (50mg/kg body weight) from day 1 to day 10.

Group IX: Animals were treated with tested compound, 6l (50mg/kg body weight) from day 1 to day 10.

Group X: Animals were treated with tested compound, 6b (125mg/kg body weight) from day 1 to day 10.

Group XI: Animals were treated with tested compound, 6j (125mg/kg body weight) day 1 to day 10.

Group XII: Animals were treated with tested compound, 6l (125mg/kg body weight) day 1 to day 10.

7.1.2.2.5. Sample collection

After 24 h of cancer cells implantation, all the animals were administered the respective dose by intraperitoneal injection on daily for ten consecutive days. Before the blood sample collection by cardiac puncture, all animals were fasted for 4 h. The blood samples were separated into two sets of micro-centrifuge tubes. From one set, serum was separated by centrifugation at 2000×g for 15 min and kept at -20˚C for further use. Heparin was added in other set of samples to study several hematological parameters (Barua et al., 2018).

7.1.2.2.6. Ascitic fluid volume and packed cells volume

The ascitic fluid was collected from peritoneal cavity of animals. The volume was determined by using graduated centrifuge tube. After centrifugation at 1000 rpm for 5 min, packed cell volume in sedimentation form.

7.1.2.2.7. Viable cells count

The ascitic fluid was taken in a WBC pipette and diluted to 100 times. The cells were then stained by 0.4% trypan blue dye and placed in a Hemocytometer. The non-viable cells took the stain while the viable cells remained transparent and counting was done accordingly.

7.1.2.2.8. Estimation of haematological parameters

7.1.2.2.8.1. Estimation of haemoglobin

20µl of heparinized blood was taken in Sahli's Haemoglobinometer and diluted with 0.1N HCl until the color look similar with the color of standard. The reading was taken directly from graduated tube in the form of g/dl (Alstead et al., 1940).

7.1.2.2.8.2. Estimation of RBC count

The blood sample was diluted to 100 times with RBC diluting fluid using Thoma pipette and mixed well accordingly. About one drop resultant mixed solution was placed in Neubauer Haemocytometer and allowed to settlement of RBC for three minutes followed by counting was done in 80 small squares under microscope (Lalee et al., 2012).

7.1.2.2.8.3. Estimation of WBC count

The blood sample was diluted to 20 times with WBC diluting fluid using Thoma pipette and mixed well. About one drop resultant solution was placed in Neubauer Haemocytometer and allowed to settlement of WBC for 3 minutes followed by counting was done under microscope in 4 large corner blocks that was further divided into 64 small squares (4×16) (Sood R et al., 1999).

7.1.2.2.9. Measurement of biochemical parameters

The collected blood samples were subjected to centrifuge to separate serum. The serum was then subjected to spectroscopic analysis to determine the serum biochemical parameters, such as glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and serum albumin, bilirubin, creatinine and urea by using automated diagnostic reagent kit according to the manufacture's protocols.

7.1.2.2.10. Survivability study

Life span of the EAC induced mice was significantly lower compared to the healthy one. To study the effect of compounds 6b, 6j and 6l on lifespan of EAC induced mice, we performed survivability assay after treatment with three compounds (6b, 6j and 6l) at their higher dose use for the *in vivo* study (125 mg/kg) and another group was treated with 5-FU (20mg/kg). To carry out the experiment, six mice were taken in each group. The survivability was determined by following equation

$$\%\ \text{survivability} = \frac{M_{treated} - M_{control}}{M_{control}} \times 100\%$$

where, $M_{treated}$ and $M_{control}$ were the number of days mice survived after treatment and survived in case of control, respectively. Kaplan–Meir survival analysis was carried out using Graph Pad Prism (GraphPad Software, La Jolla, CA).

7.1.2.2.11. Histopathological Study

On 11th day, liver tissues were dissected out from each group of sacrificed animal and preserved in formalin solution immediately. The harvested tissues were embedded in paraffin wax followed by cut into thin sections by using microtome instrument. The sections were then placed on clean glass slides to counter stain with hematoxylin and eosin (H&E) where hematoxylin stains the nucleus with blue color whereas cytoplasm stained with eosin which was red in color. After staining, the glass slides containing liver sections of different groups were subjected to take images under light microscope.

7.1.3. Statistical analysis

Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. $P < 0.01$ was considered as statistically significant whereas $P > 0.05$ was considered to be statistically not significant.

7.2. Results and discussion

7.2.1. *In vitro* assay

Among the other types of cancer, breast cancer is common among women. According to world health organization (WHO), each year about 2.1 million women affected by it globally. Recent reports of 2018 suggested that about 0.6 million women died from breast cancer which is nearly 15% of all cancer-related deaths among women. The statistical data itself indicated the necessity to develop new anticancer agents. By accepting this challenge scientist from all over the globe have been tried to develop new leads over decades and have found 1,2,4-triazole and hydroxamic acid derivatives have a huge potency to be an anti-breast cancer agent. We have recently synthesized and identified twelve derivatives of 4-amino-5-((aryl substituted)-4h-1, 2, 4-triazole-3-yl. The synthesized compounds were explored for their *in vitro* anticancer activity using MTT assay against three human cancer cell lines, among them two were breast cancer cell lines like, MCF-7, MDA-MB 231 whereas, another one was colon cancer cell line i.e. HCT 116. It was found that majority of the tested compounds exhibit anticancer activity with IC_{50} values ranging from 3.74±0.79 to 168.73±7.79µg/ml (Table 6). Some of them exhibited promising anticancer activity against the three cancer cell lines.

To compare the effect of the tested compounds on highly invasive (MDA-MB-231), poorly invasive (MCF-7) breast cancer cells, and human colon carcinoma cells (HCT 116) we treated these cells with the synthesized compounds (0–320µg/ml) for 24 hours and percent inhibitions were determined. From which IC_{50} values were calculated and tabulated as table 6.

Table 6 IC_{50} values of synthesized compounds against cell line MCF-7, MDA-MB-231 and HCT 116.

Tested compounds	IC_{50} (µg/ml)		
	MCF-7	**MDA-MB-231**	**HCT 116**
6a	23.77 ± 4.28	47.15 ± 1.48	10.82 ± 1.68
6b	15.85 ± 3.52	41.82 ± 1.20	10.408 ± 0.75
6c	22.27 ± 4.68	39.77 ± 1.11	14.69 ± 4. 37
6d	20.13 ± 2.88	38.49 ± 0.46	6.58 ± 1.58
6e	90.61 ± 9.56	98.09 ± 4.22	168.73 ± 7.79
6f	74.89 ± 9.6	92.19 ± 9.47	85.14 ± 4.76
6g	58.23 ± 6.29	92.00 ± 4.43	8.13 ± 0.39
6h	34.21 ± 5.99	86.27 ± 8.28	6.52 ± 1.33
6i	26.27 ± 4.15	38.31 ± 4.27	8.52 ± 1.57
6j	19.02 ± 2.08	37.02 ± 4.35	3.74 ± 0.79
6k	23.07 ± 3.63	44.59 ± 2.48	19.24 ± 2.22
6l	16.69 ± 1.60	43.80 ± 2.16	19.18 ± 3.7 2

Data expressed as Mean ± SD, n=3

From table 6 it could be found that all of the tested compounds have shown better activity (according to their IC_{50} values) against MCF-7 breast cancer cell lines rather than the triple negative breast cancer cell line, MDA-MB-231. Among them, compound 6b, 6j and 6l have shown better inhibition against MCF-7 with the IC_{50} values of 15.85 ± 3.52, 19.02 ± 2.08 and 16.69 ± 1.60µg/ml, respectively. Besides the activity against two different breast cancer cell lines, the tested compounds have shown superior inhibition against HCT 116 with a minimum

IC_{50} of 3.74 ± 0.79µg/ml for compound 6j. It could be very interesting to found that increasing the linear chain length at R^2 position made the compounds more active i.e. compounds 6b, 6d, 6f, 6h, 6j, 6l were more active than compounds with methyl substitution at R^2 position like compound 6a, 6c, 6e, 6g, 6i, 6k. Which in turn indicated that the linker group (R^2) of compound 6b, 6d, 6f, 6h, 6j, 6l was probably beneficial to its activity. The tested compounds were designed and synthesized with two points of diversity i.e. variation of substituent on the surface recognition moiety (phenyl ring) and variation of the linker group. However, the better anticancer activity of compound 6b, 6j and 6l among the twelve tested compounds against MCF7 reflects the importance of the substituent like pyridyl, *p*-nitrobenzene and *m*-nitrobenzene on the surface recognition moiety played important role in their biological activity. The results could also be justified by the research done by Saha et al where they have synthesized hydroxamic acid linked 5-aryl substituted 4-amino-1,2,4-triazole analogues and found to have similar binding interaction with receptor, HDAC8, like standard SAHA (Saha et al., 2010). Hassan et al also demonstrated that aryl substitution at the 5-position of 1,2,4,-traizole ring could develop potent anticancer agent (Hassan et al., 2018).

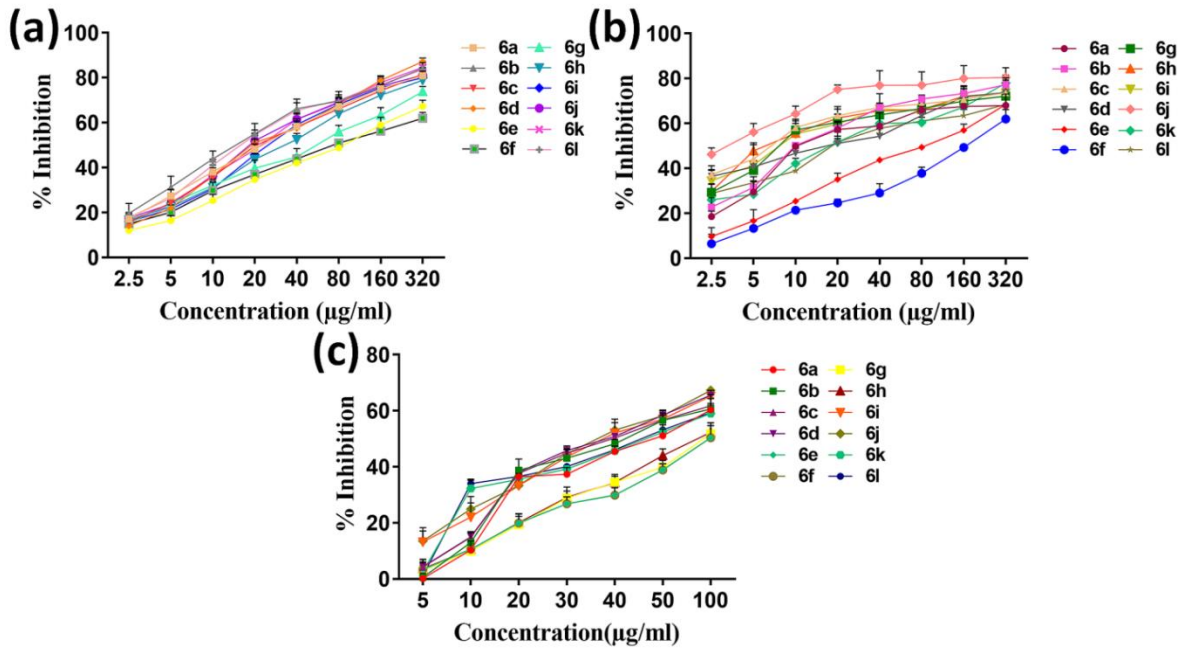


Fig 38. Percentage inhibition of compounds 6(a-l) against **(a)** MCF-7 cell line, **(b)** MDA-MB-231 cell line and **(c)** HCT 116 cell line.

Considering the *in vitro* anti cancer data generated for tested compounds against the breast cancer cell line like MCF-7, compound 6b, 6j and 6l were further selected to evaluate their *in vivo* anticancer activity against EAC induced mice model.

7.2.2. *In vivo* experiment

7.2.2.1. Ascitic fluid, packed cells and viable cell count

EAC is a very rapid growing and produces liquid tumor in abdominal region. This type of carcinoma is very aggressive in nature results elevated vascular permeability, higher cellular migration and formation as well as accumulation of ascitic fluid. As the ascitic fluid act as a direct source of nutrition for the tumor cells, it plays a very important role in tumor formation. So, minimizing the volume of ascitic fluid, packed cells as well as viable cell count indicated the efficacy of a compound as an anticancer agent. In this experiment, compounds 6b, 6j and 6l showed significant dose dependent reduction of ascetic fluid volume, packed cell volume, viable

cell count compared to EAC control animals (Fig 38). When the volume of ascitic fluid upon the treatment of compounds 6b, 6l and 6j compared with the standard it could be found that compound 6b at a concentration of 125mg/kg have shown no significant differences, indicated the potency to decrease the volume of ascitic fluid compare to that of the standard. Similar results have been found in case of packed cell volume and viable cell count, compound 6b (125mg/kg) showed comparable potency with standard.

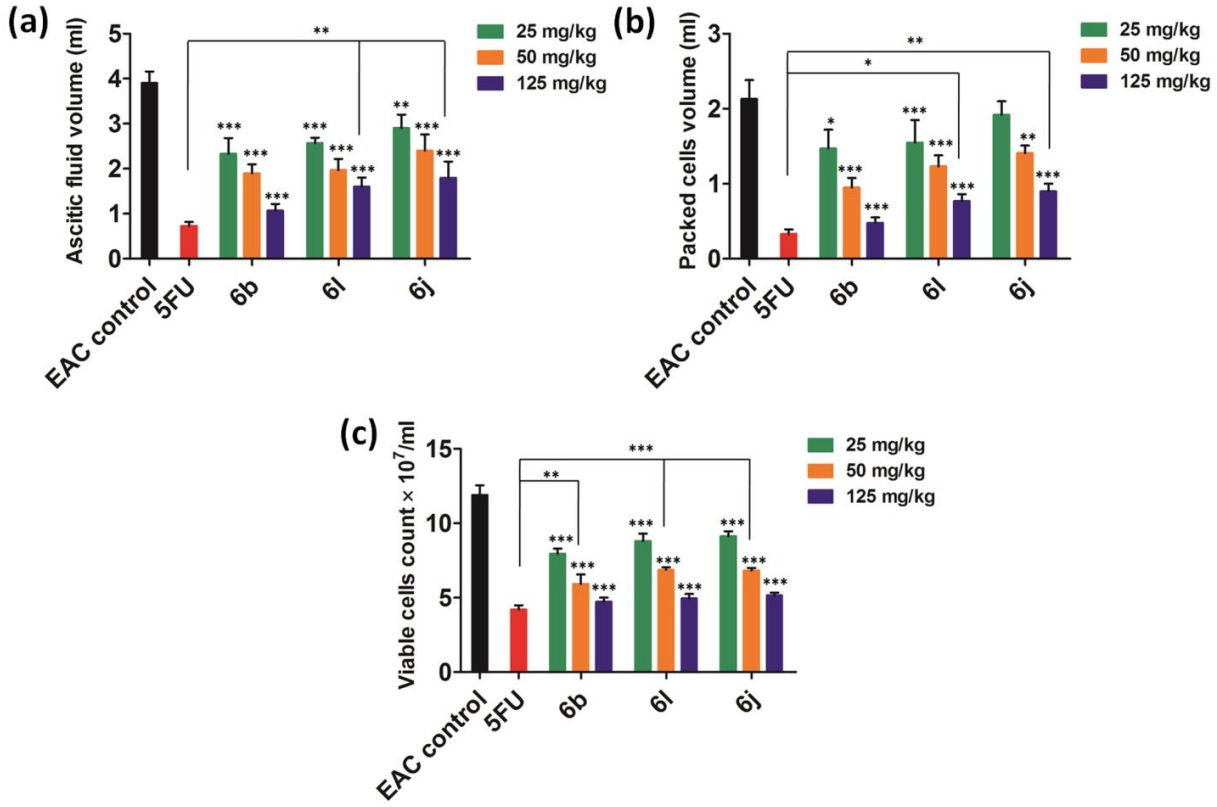


Fig. 39. (a) Ascitic fluid volume; **(b)** Packed cell volume and **(c)** viable cell count was carried out for compounds 6b, 6j and 6l at different concentrations like 25, 50 and 125 mg/kg body weight. 5-FU was used as standard. Data expressed as Mean ± SD, n=3; significant difference was calculated against control; *** denote $p<0.001$, ** denote $p<0.01$, * denote $p<0.05$

7.2.2.2. Hematological parameters

When hematological parameters were measured, notable reduction of hemoglobin level (10.82±0.42 g/dl), RBC counts (5.41±0.47) and elevated WBC counts (11.81±0.51) were observed in case of EAC control group of animals (Table 7). The treated group containing compounds 6b, 6j and 6l showed dose dependent increase in hemoglobin (14.44±0.43, 12.90±0.42 and 14.17±0.51g/dl), RBC (8.17±0.24, 6.92±0.19 and 8.10±0.38) counts and decreased in WBC counts (6.70±0.20, 7.82±0.24 and 6.30±0.32) nearer to normal value. Like the result obtained for ascitic fluid volume, packed cell volume and viable cell count of compound 6b, here also a similar trend could be observed at the dose of 125mg/kg. From the statistical analysis comparing the results of control it was clearly observed that compound 6b and 6l have shown almost negligible significant difference which indicated the fact that pyridyl and m-nitrobenzene moiety at R^1 position was important to exert anticancer potency of the tested compounds.

Table 7 Effect of compounds 6b, 6j and 6l on different blood parameters of EAC cells bearing mice showed reduction in all the blood parameter comparable to normal mice.

	Hemoglobin (g/dL)	**RBC (/ml)**	**WBC (/ml)**
Control	15.82±0.62	8.78±0.44	6.79±0.41
EAC control	10.82±0.42	5.41±0.47	11.81±0.51
6b (25 mg/kg)	11.137±0.35***	6.02±0.34**	9.22±0.24***
6b (50mg/kg)	12.03±0.45***	6.71±0.14**	8.65±0.22***
6b (125 mg/kg)	14.44±0.43*	8.17±0.24	6.70±0.20
6l (25 mg/kg)	10.97±0.38***	5.51±0.32**	10.14±0.51***
6l (50mg/kg)	11.36±0.54***	6.65±0.61**	8.85±0.27***

6l (125 mg/kg)	14.17±0.51*	8.10±0.38	6.30±0.32
6j (25 mg/kg)	10.95±0.13***	5.55±0.12**	9.98±0.27***
6j (50mg/kg)	11.59±0.39***	6.14±0.68**	8.93±0.37***
6j (125 mg/kg)	12.90±0.42***	6.92±0.19***	7.82±0.24*

Data expressed as Mean ± SD, n=3; significant difference was calculated against control;

***** denote p<0.001, ** denote p<0.01, * denote p<0.05**

7.2.2.3. Biochemical parameters

SGPT, SGOT, creatinine, bilirubin and urea level were significantly increased in the serum after development of ascetic carcinoma in compared to the normal mice whereas albumin level were significantly reduced. Liver and kidney toxicities levels almost back to normal after treatment with compounds 6b, 6j (dose of 125mg/kg) in mice having ascetic carcinoma in compared to the normal mice (Table 8). It could be observed from table 8 that SGPT, SGOT, creatinine, urea and bilirubin level of compound 6b markedly decreased than compound 6j at the dose of 125mg/kg body weight. Although there is no significant different in the result obtained from compound 6b and 6l (125mg/kg), the change of biochemical markers compare to the control is more significant than 6l to 6b at even higher dose.

Table 8 Toxicity testing in serum, from EAC cells bearing mice, after treatment with different compounds 6b, 6j, 6l showed reduction of both hepato and nephro-toxicity in the treated groups which were comparable with normal mouse.

	SGPT (U/L)	**SGOT (U/L)**	**Albumin (g/dl)**	**Creatinine (mg/dl)**	**Urea (mg/dl)**	**Bilirubin (mg/dl)**
Control	44.78±4.67	147.78±8.54	3.05±0.35	0.39±0.02	40.12±2.83	0.10±0.01
EAC control	89.68±8.33	294.77±11.32	1.55±0.21	0.83±0.07	63.47±6.93	0.36±0.05

6b (25mg/kg)	64.63±5.93**	187.57±10.20**	2.08±0.15**	0.52±0.03**	50.03±3.19*	1.98±0.28***
6b (50mg/kg)	58.78±5.48*	174.95±14.14	2.55±0.16	0.45±0.05	47.77±5.92	1.78±0.25***
6b (125mg/kg)	55.43±5.07	170.45±15.09	2.78±0.31	0.42±0.04	44.52±4.03	1.62±0.29
6l (25mg/kg)	74.58±5.39***	199.17±9.00***	2.20±0.20**	0.59±0.05***	55.28±5.18**	2.68±0.34***
6l (50mg/kg)	65.78±4.69**	190.32±11.44**	2.30±0.58	0.50±0.06	50.12±2.76	2.32±0.34***
6l (125mg/kg)	60.38±4.23*	174.50±14.48	2.45±0.23	0.45±0.05	46.75±5.23	2.15±0.41
6j (25mg/kg)	77.03±5.44***	212.18±8.82***	1.90±0.15**	0.63±0.03***	56.90±3.28**	2.83±0.29***
6j (50mg/kg)	73.33±4.52***	197.83±9.54**	2.05±0.15*	0.56±0.04**	50.67±3.38*	2.52±0.30***
6j (125mg/kg)	67.63±6.03**	178.78±14.78	2.37±0.33	0.47±0.05	48.07±4.94	2.27±0.42

Data expressed as Mean ± SD, n=6; significant difference was calculated against control; * denote p<0.001, ** denote p<0.01, * denote p<0.05**

7.2.2.4. Survivability study

It could be found from fig 40a that survivability has seriously been affected by the EAC induction. The effect of the dose have been used to treat the animals on their reduced survivability was the main objective of this particular experiment and found very interesting results. Compare to the effect of 5-FU treatment on the percent survival, the tested compounds have shown promising activity, specially compound 6b. From fig 40b it could be clearly observed that the rate of survivability has been significantly increased in case of compound 6b compare to the other tested compounds, even from the standard. It was found that 5-FU elevated the life span of the EAC bearing mice to almost 191%, whereas compound 6b at its higher experimental dose of 125mg/kg body weight, elevated it to 205%. Though the life span elevation

due to the treatment of compound 6b was statistically non-significant compare to the standard still it could be said that it produces similar effectiveness among the among the other two tested compounds 6l and specially 6j. The percent survivability of compound 6j was markedly declined compare to the compound 6b which could be justified by the results obtained in table 7 and table 8**.** From table 7 it could be found that the hemoglobin, RBC and WBC count weren't significantly increased compare to the compound 6b and the scenario was same in case of biological markers presented in table 8.

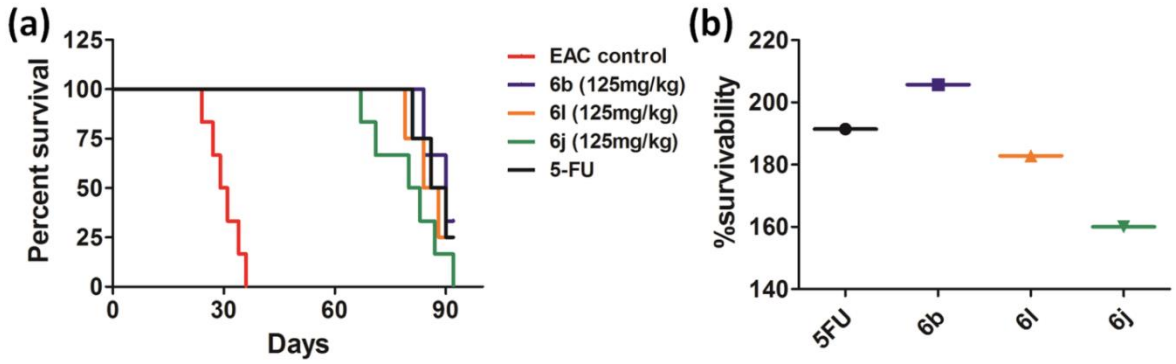


Fig 40. (a) Kaplan–Meir survival plot, **(b)** comparative study on percent survivability

7.2.2.5. Histology

Fig 41 showed the effect of treatment on the liver, as liver is the most effected organ in EAC bearing mice. In this study we not only have shown the effect of different compounds but also have shown the effect of different doses on the particular organ. Fig 41a represents the liver section of normal control. Normal hepatocytes with vesicular nuclei could be observed with normal anatomy of sinusoid capillaries. Hepatocytes were evenly separated by sinusoids. Whereas liver section of EAC bearing mice (Fig 41b) showed vacuolated hepatocytes along with steatosis. Dilated sinusoids have also been found in EAC control. Effect of different doses of compound 6b could be observed in fig 41d-41f. From fig 41f it could be found that the hepatocytes recover their normal anatomy, even distribution of sinusoids around the hepatocytes

could be observed like control. From fig 41i and 41l it could be found that recovery of liver was happened but not the extent as fig 41f because vacuolated hepatocytes and large sinusoids were still present which indicated that compound 6b (125mg/kg) was more effective and safer anticancer agent than compound 6l and 6j. From the histopathological image it could also be found that compound 6b (fig 41f) was as effective as standard drug 5-FU (fig 41c) in respect to recover the vital organ, liver.

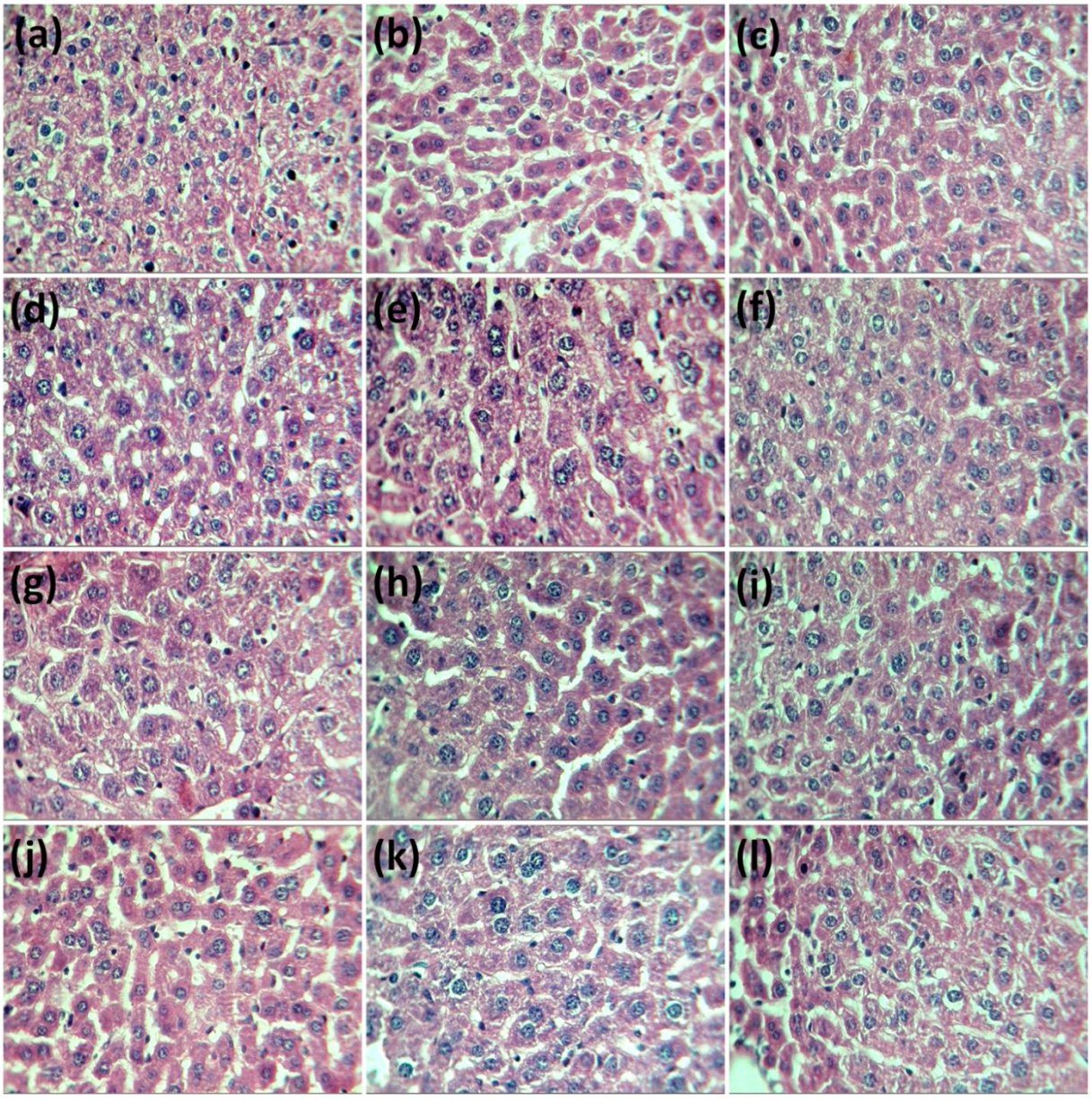


Fig 41. H&E stained liver section of mice; **(a)** untreated control, **(b)** EAC control, **(C)** 5-FU treated (20mg/kg), **(d)** treated with 6b (25mg/kg), **(e)** treated with 6b (50mg/kg), **(f)** treated with 6b (125mg/kg), **(g)** treated with 6l (25mg/kg), **(h)** treated with 6l (50mg/kg), **(i)** treated with 6l (125mg/kg), **(j)** treated with 6j (25mg/kg), **(k)** treated with 6j (50mg/kg), **(l)** treated with 6j (25mg/kg).

7.3. Conclusion

It could be concluded that the synthesized compounds have shown promising anticancer activity *in vitro* as well as *in vivo*. Compound 6b, 6j and 6l were selected for the *in vivo* study and from the results it could be concluded that compound 6b was best among other derivatives. The effectiveness could be explained by the addition of pyridyl group at the surface recognition moiety (R^1 position). Molecular mechanism of the therapeutic potency of the synthesized compounds still to investigate. Evaluation of the signaling pathways could be done in future to elicit the anticancer mechanism of the synthesized compounds. This chemical frame work could also lead to produce compound with more efficacy with less toxicity.

Chapter 8

8. Antileishmanial evaluation

Leishmaniasis is a tropically neglected parasitic disease that is commonly seen in African regions, Central and South America, Southern Europe, the Middle East, and the Indian subcontinent (Khattab et al., 2017). According to World Health Organization (WHO), an estimation of 0.3 million new cases of visceral leishmaniasis is reported annually and the mortality rate is 20000 per year. Leishmanial parasite can cause a wide spectrum of diseases like visceral, cutaneous, mucocutaneous and diffused cutaneous leishmaniasis (Khattab et al. 2017). 1 million cases of cutaneous leishmaniasis are reported annually, whereas over1 billion people living in endemic areas are at risk of infection. Along with drug resistances, HIV co-infection made the disease most fatal one (Lindoso et al., 2016). The existing chemotherapeutics miltefosine, paromomycin, sodium stibogluconate, meglumine antimoniate, amphotericin B are drugs used for the treatment of leishmaniasis but they possess toxicity along with side-effects (Ghosh et al., 2016) To solve these problems there is an urgent need for novel, efficacious, less toxic and safe drug candidates in the pipeline (Walker et al., 2012 and Bekhit et al., 2015). To address the concern, we develop a series of novel 5-substituted-4-amino-1,2,4-triazole linked hydroxamic acid derivatives. Assembling the different pharmacophores in a single frame always leads to compounds having fascinating biological profile (Crumplin et al., 1975) Triazole is one of the pharmacophores in the synthesized compounds. Chemical compounds possess triazole as a core; always play a significant role in the field of medicinal chemistry. Triazole is well known for its antimicrobial property and widely used to treat microorganisms associated diseases (Jain et al., 2013) Besides the antibacterial (Prasad et al., 2009 and Foroumadi et al., 2003) and antifungal (Ram et al., 1974; Reddy et al., 1990 and Hiremath et al., 1989) activity; 1,2,4-triazole possess antileishmanial activity (El-Saghier et al., 2019). Moreover, ravuconazole, albaconazole,

and isavuconazole (triazole derivatives) are currently under investigation in clinical trials (Mindt et al., 2006).

So, considering the above fact we have designed and synthesized some novel 5-substituted-4-amino-1,2,4-triazole linked hydroxamic acid derivatives and evaluate their activity against wild type, field type and drug resistant *L. donovani* amastigotes.

8.1. Materials and Methods

8.1.1. Materials

Amphotericin B (AmB), paromomycin (PMM), medium 199, fetal bovine serum and RPMI 1640 were purchased from Sigma- Aldrich, India. Fluorescin isothiocyanate 98% (FITC), was procured from Himedia Laboratories. Sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH) was procured from Merck, India. Sodium stibogluconate (SSG) was a generous gift from Albert David Ltd. (Kolkata, India). Dichloromethane (DCM) was procured from Merck, India. All other chemicals used were analytical grade.

8.1.2. Methods

8.1.2.1. Parasite culture

Medium 199 supplemented with 2 mM L-glutamine, 100 mg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, 20 mM HEPES and 10% heat inactivated fetal bovine serum were used to culture *L. donovani* AG83 promastigotes at 25°C. MMA/20 (medium for axenic amastigote) pH 5.5 was used to derive the axenic amastigote form of *L. donovani* from the promastigotes. Parasite culture was maintained at 37°C in a 5% CO_2 incubator with a sub-passaging period of every five days (Ghosh et al., 2017).

8.1.2.2. Development of drug resistant amastigote strains

The development of drug resistant *L. donovani* strain was done as Ghosh et al (Ghosh et al., 2017). Briefly, the promastigotes of AG83 wild-type were cultured along with a drug concentration equivalent to theirIC_{50} value of that drug against the strain. After three subcultures, the drug concentrations were amplified in such a manner that the survived cell population was around 80% for each of the batches. When almost 90% cells of the initial cell population were damaged, the survived strains were cultured further on medium 199 agar plates in the presence of that particular drug concentration. After that a single colony was isolated and cultured in liquid medium 199. Drug pressure was withdrawn and the stability was verified at the first, second and fourth months.

8.1.2.3. Isolation of peritoneal macrophages

Into the peritoneal cavity of BALB/c mice (either sex, weigh 20-25 g) 1.5-2 ml sterile thioglycolate medium was injected. 5 days afterwards, animals were sacrificed by cervical dislocation and 4-5 ml of cold RPMI-1640 medium was injected through intra-peritoneal injection. After gentle massaging of the peritoneal part, 2-3 ml of peritoneal exudates were collected by the same syringe and centrifuge it for 15 min at 5000 rpm. Cells were collected and non-specific esterase staining was done to confirm that almost 60-80% of total cells were macrophages.

Trypan blue exclusion assay was performed to determine the viability of the isolated macrophages. Briefly, 0.2 ml of isolated cells were aseptically taken into an eppendorf tube. After 3 min incubation at room temperature equal volume of trypan blue (0.4% w/v) was added. Hemocytometer was used to count the cells under microscope. Viable cells as well as non- viable cells were counted separately and it was found that 90-95% macrophages were viable.

8.1.2.4. In vitro growth inhibition study

50% inhibitory concentrations (IC_{50}) of synthesized compounds were determined from the *in vitro* growth inhibition study. Cell count technique was employed to study the growth inhibition of axenic amastigotes of *L.donovani* AG83 wild type, GE1 field type, SSG resistant and PMM resistant strains. 10^5 amastigotes of different strains were seeded in a 96 well plates. Tested compounds and AmB were added at their predetermined concentration and place. Cell counts were taken by hemocytometer after incubation period of 72h in 5% CO_2 incubator at 37°C. The amastigote counts of control and tested compound treated was compared and assumed that the counts of control mean 100% growth. Percent of growth inhibition was calculated by the following equation (Nahar et al., 2009).

% of inhibition = [(count of control- count of treated)/count of control] ×100

8.1.2.5. Drug susceptibility assay of intracellular amastigotes

To carry out drug susceptibility assay of intracellular amastigotes sterile cover slips were placed into the wells. 0.5 ml of RPMI-1640 medium containing macrophages at a concentration of 4×10^5 cells per ml was seeded into each well and incubated at 37°C for 2 h in 5% CO_2 incubator. After that 0.5 ml of warm supplemented RPMI-1640 medium replaced the old media. 0.5 ml of medium containing amastigotes at a concentration of 4×10^6 amastigotes per ml was added into the wells and kept for 4 h at 37°C in the 5% CO_2 incubator for internalization of amastigotes into the macrophages. Old media was replaced by fresh RPMI-1640 medium and tested compounds and AmB at appropriate concentrations were added in triplicate at predetermined wells and kept for 72 h in 5% CO_2 incubator at 37°C. After 72 h media was removed carefully and cover slips were removed, fixed, air dried and stained with Giemsa. Numbers of amastigotes in 100 macrophage cells were counted under microscope for each cover slip. The assay results were

calculated by calculating the ratio between the infection proportions of control and treated macrophage.

8.1.2.6. *In vitro* cytotoxicity assessment

MTT assay was carried out to determine the cytotoxicity of synthesized compounds and standard drug AmB, by determining the mitochondrial activity colorimetrically. 96 well plate was used to carry out the assay. Supplemented RPMI-1640 medium having macrophages at a concentration of 10^4 cells per ml was seeded to each well. All the synthesized compounds were added in triplicates at their predetermined concentrations and incubated at 37°C in 5% CO_2 incubator for 24 h. Media was replaced with fresh media containing MTT (10 µl of MTT for each well having a concentration of 0.5mg/ml) was added to each well and kept for 4 h at 37°C in 5% CO_2 incubator. 0.2 ml of DMSO was added to wells to dissolve formazon crystals after removing the media carefully. The absorbance was measured at 570 nm by microplate readers. The results were determined by calculating the percent of reduction in cell viability compared to the control.

8.1.2.7. In silico target binding study

AutoDock vina (Trott et al., 2010) using the Lamarckian genetic algorithm (LGA) for the prediction of binding affinity and searching for the optimum binding site together with the AutoDock Tools (ADT) were employed to set up and perform blind docking calculations of the triazole derivatives binding to substrate binding site of respective enzymes. The protein domain of *Leishmania donovani* (strain BPK282A1) (Pdb id: 5FEA) domain Swapped Bromo domain from *Leishmania donovani*; (Podust et al., 2001) was obtained from the Protein Data Bank (www.rcsb.org/pdb) (Rose et al., 2012) at a resolution of 1.60 Å was constructed using Autodock4 package to study the target binding interaction of all the compounds under study. The

coordinates of tested compounds were taken from their 2D structures sketched from ACD ChemSketch Freeware and converted to the PDB format using openbabel (O'Boyle et al., 2011). The target (respective proteins) and the ligand (triazoles) files were prepared using AutoDock Tools. The water molecules were deleted and polar hydrogen atoms and Gasteiger charges were added to both the target molecule and the ligands. All other bonds were made rotatable. The target was saved in pdbqt format and a configuration file was created. Later docking was run. Autodock generated 9 possible binding conformations for each ligand and the root-mean-square (RMS) cluster tolerance was set to 2.0 Å in each run. The binding mode and interactions were analyzed for the significant conformers of the compounds under study. All calculations were performed in a Dell system (3.4 GHz processor, 2GB RAM, 320 GB Hard disk operating system. For each of the docking cases, the lowest energy docked conformation, according to the Autodock scoring function, was selected as the binding mode. Visualization of the docked pose was done by using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC) molecular graphics program.

Docking was processed with setting of the grid sizes for 5FEA 26, 26, and 30 along the X, Y and Z axes with 1Å spacing which covered all the active residues. In a similar way the grid center of each target was set for 5FEA 1.75, 38.95 for the protein.

8.1.3. Statistical analysis

Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. $P < 0.01$ was considered as statistically significant whereas $P > 0.05$ was considered to be statistically not significant.

8.2. Results and discussion

Table 9 shows the *in vitro* susceptibility of synthesized compounds against *L. donovani* AG83 wild type, SSG-R, PMM-R and GE1 field isolated axenic amastigotes. Resistance index (RI) was calculated for all the tested compounds. RI is the ratio between IC_{50}value of AG83 phenotype generated at maximum drug pressure and IC_{50}value of wild-type strain of any particular compound. It was observed from Fig 3A that among the twelve tested compounds, compound 6g, 6h, 6k and 6l showed higher RI values against SSG-R (1.51, 1.42, 1.57 and 1.49, respectively) and PMM-R strains (1.69, 1.54, 1.47 and 1.43, respectively). However, in comparison with other compounds the values were not significantly higher. For all the compounds the RI values were close to 1 which indicated that the synthesized compounds have similar kind of effect against drug resistant (SSG-R, PMM-R) as well as wild type AG83 and GE1 field type *L. donovani* strains.

Table 9: IC_{50} values of the synthesized compounds against *Leishmania donovani* AG83 wild type, drug resistant and GE1 field type axenic amastigotes[a]

Compounds	IC_{50} (mean ± SD, n = 3) µg/ml						
	Axenic AG83 evaluation model						
	Wild type	SSG-R	RI[f]	PMM-R	RI	GE1	RI
6a	92.34±2.21 [b]	98.88±3.77[c]	1.07	94.63±3.43[d]	1.03	110.22±5.12[e]	1.19
6b	107.65±2.27 [b]	125.34±2.71 [c]	1.16	117.46±3.01 [d]	1.09	142.35±4.87 [e]	1.32
6c	65.91±4.02 [b]	84.08±4.08 [c]	1.28	90.73±4.49 [d]	1.38	72.98±2.86 [e]	1.11
6d	69.18±2.48 [b]	76.65±3.02 [c]	1.11	80.96±2.50 [d]	1.17	74.63±3.64 [e]	1.08
6e	98.58±4.54 [b]	117.31±2.89 [c]	1.19	109.70±5.44 [d]	1.11	113.46±3.45 [e]	1.15
6f	104.88±3.43 [b]	124.81±3.15 [c]	1.19	112.48±4.28 [d]	1.07	120.81±3.53 [e]	1.15
6g	61.08±3.64 [b]	92.26±2.02 [c]	1.51	103.55±3.09 [d]	1.69	71.65±2.40 [e]	1.17

6h	62.16±3.81 [b]	88.11±4.00 [c]	1.42	95.76±4.21 [d]	1.54	72.32±4.19 [e]	1.16
6i	69.33±2.07 [b]	78.92±3.37 [c]	1.14	75.18±3.39 [d]	1.08	75.62±3.75 [e]	1.09
6j	67.96±3.44 [b]	76.53±3.32 [c]	1.13	68.27±2.36 [d]	1.01	70.15±3.61 [e]	1.03
6k	69.22±2.95 [b]	108.45±4.48 [c]	1.57	101.42±2.81 [d]	1.47	78.28±3.84 [e]	1.13
6l	75.36±3.07[b]	112.13±3.55 [c]	1.49	107.37±3.38 [d]	1.43	82.34±3.34 [e]	1.09
AmB	0.19±0.03	0.22±0.02	1.15	0.25±0.04 [d]	1.31	0.20±0.04 [e]	1.05

[a] Assay method was described in methods section.

[b] P< 0.01 significant compared to AmB

[c]P< 0.01 significant compared to AmB

[d]P< 0.01 significant compared to AmB

[e]P< 0.01 significant compared to AmB

[f] RI, Resistance Index was IC50 of AG83 phenotype generated at maximum drug pressure/IC50 of wild-type

The critical balance between safety and efficacy always play the crucial role in development of new drug candidates. To find out the balance between safety and efficacy of the drug substances selectivity index (SI) was employed. SI indicates the maximum exposure of the drug which produce desire efficacy with no toxicity. In the present study, SI value was expressed as a ratio between the 50% cellular cytotoxic concentration (CC_{50}) of a compound and 50% inhibitory concentration (IC_{50}) of that particular compound against any strains used for the experiment and it was found from the previous studies that when SI values is more than 10 it indicates the promising activity of the drug substances (Mondal et al., 2013).

Table 10: IC_{50} values synthesized compounds against *Leishmania donovani* AG83 wild type, drug resistant and GE1 field type cellular amastigotes[a]

Compounds	IC_{50} (mean ± SD, n = 3) µg/ml								CC_{50} (µg/ml) (Macrophage cells) (mean ± SD, n = 3)
	Cellular AG83 evaluation model								
	Wild type	SI[g]	SSG-R	SI[g]	PMM-R	SI[g]	GE1	SI[g]	
6a	86.52±3.32[b]	10.86	93.45±3.87[c]	10.05	88.32±4.00[d]	10.64	92.54±3.76[e]	10.16	940.0±16.94[f]
6b	94.44±3.39[b]	9.18	110.26±4.10[c]	8.41	102.66±4.40[d]	9.03	102.28±2.93[e]	9.07	927.2±23.08[f]
6c	62.85±4.78[b]	84.98	78.64±3.32[c]	67.91	81.56±4.67[d]	65.48	69.12±3.55[e]	77.27	5340.7±54.64[f]
6d	65.15±4.52[b]	78.98	70.32±3.20[c]	73.17	72.18±3.15[d]	71.28	71.54±4.14[e]	71.92	5145.4±34.45[f]
6e	89.96±3.63[b]	99.38	110.26±3.86[c]	81.09	98.14±2.11[d]	91.10	107.22±4.55[e]	83.39	8940.6±59.69[f]
6f	95.42±3.91[b]	93.02	116.65±3.59[c]	76.09	104.25±5.40[d]	85.13	115.86±4.77[e]	76.61	8875.5±69.46[f]
6g	55.68±3.21[b]	43.92	84.28±4.62[c]	29.02	88.56±4.15[d]	27.61	63.22±3.76[e]	38.68	2445.5±31.40[f]
6h	56.24±4.15[b]	43.15	78.16±4.46[c]	31.05	83.08±2.27	29.21	65.72±4.74[e]	36.93	2426.8±35.76[f]
6i	65.46±2.77[b]	23.82	68.36±4.19[c]	22.81	70.84±4.17[d]	22.01	67.25±3.90[e]	23.19	1559.5±24.19[f]

6j	62.88±3.27 [b]	26.24	66.42±4.09 [c]	24.84	63.28±3.53 [d]	26.07	67.46 ±4.15 [e]	24.46	1650.0±38.87 [f]
6k	63.64±3.10 [b]	79.28	97.25±3.04 [c]	51.88	92.14±4.58 [d]	54.76	70.65 ±6.38 [e]	71.41	5045.4±28.63 [f]
6l	70.18±4.05 [b]	69.61	102.54±4.8 8 [c]	47.65	99.27±2.20 [d]	49.22	76.37 ±2.90 [e]	63.97	4885.6±30.60 [f]
AmB	0.14±0.02	79.28	0.17±0.05	65.29	0.19±0.03	58.42	0.15± 0.04	74.00	11.1±1.04

[a] Assay method was described in methods section.

[b] P< 0.01 significant compared to AmB

[c]P< 0.01 significant compared to AmB

[d]P< 0.01 significant compared to AmB

[e]P< 0.01 significant compared to AmB

[f]P< 0.01 significant compared to AmB

[g] SI, Selectivity Index was CC_{50} of the drug or tested compound in macrophages/ IC_{50} of the drug or tested compound

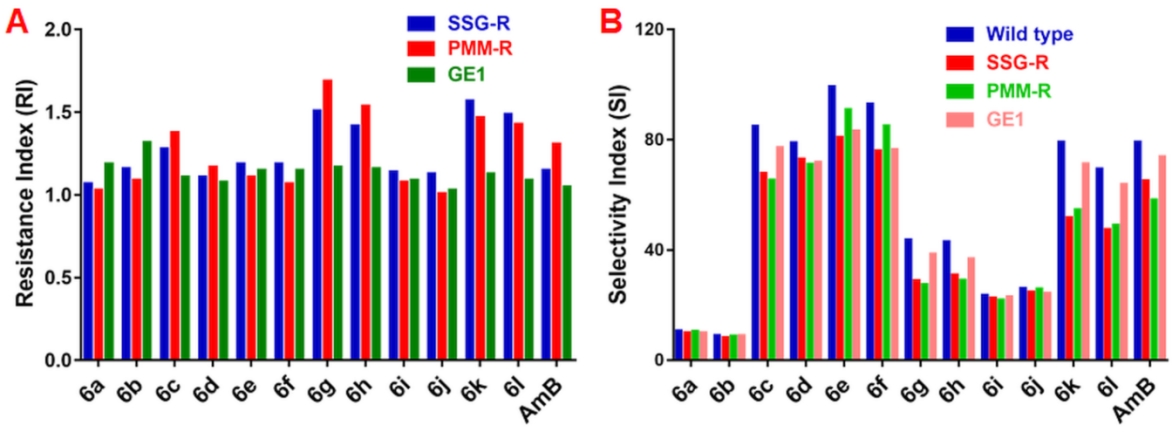


Fig 42. Comparative study of resistance index of the synthesized compounds

From Table 10 and Fig 42B it could be observed that compound 6e have higher SI values against AG83 wild type (99.38), SSG-R (81.09), PMM-R (91.10) and GE1 field type (83.39) strains followed by compound 6f even compare to the SI values of amphotericin B (AmB) for respective strains. According to SI index, the synthesized compounds were oriented in a descending manner as per the antileishmanial efficacy like,

Wild type: 6e> 6f> 6c> 6k> 6d> 6l> 6g> 6h> 6j> 6i> 6a> 6b

SSG-R: 6e> 6f> 6d> 6c> 6k> 6l> 6h> 6g> 6j> 6i> 6a> 6b

PMM-R: 6e> 6f> 6d> 6c> 6k> 6l> 6h> 6g> 6j> 6i> 6a> 6b

GE1: 6e> 6c> 6f> 6d> 6k> 6l> 6g> 6h> 6j> 6i> 6a> 6b

The data obtained from antileishmanial evaluation revealed that compound 6e was the most potent than the rest of the compounds considered for the antileishmanial study. The affinity towards the species may be attributed to the essential chemical features, i.e. ortho mercapto phenyl at position 5, the other hydroxamic acid side chain at position 3 and their relative position within the molecule.

It could be proposed from the antileishmanial efficacy data of all the synthesized compounds that presence of o-mercaptophenyl at 5^{th} position of the 1,2,4-triazole ring produced higher antileishmanial activity followed by 4-aminophenyl and 3- nitrophenyl at the same position. From the obtained result it was also observed that the antileishmanial efficacy of the synthesized compounds was affected most of the cases by increasing the hydrocarbon chain length attached directly to the thio ether group. Whereas, the o-mercaptophenyl linked triazole exhibited less cytotoxicity. 3-nitrophenyl and 4-aminophenyl were the few other, showed similar extent of cytotoxic property.

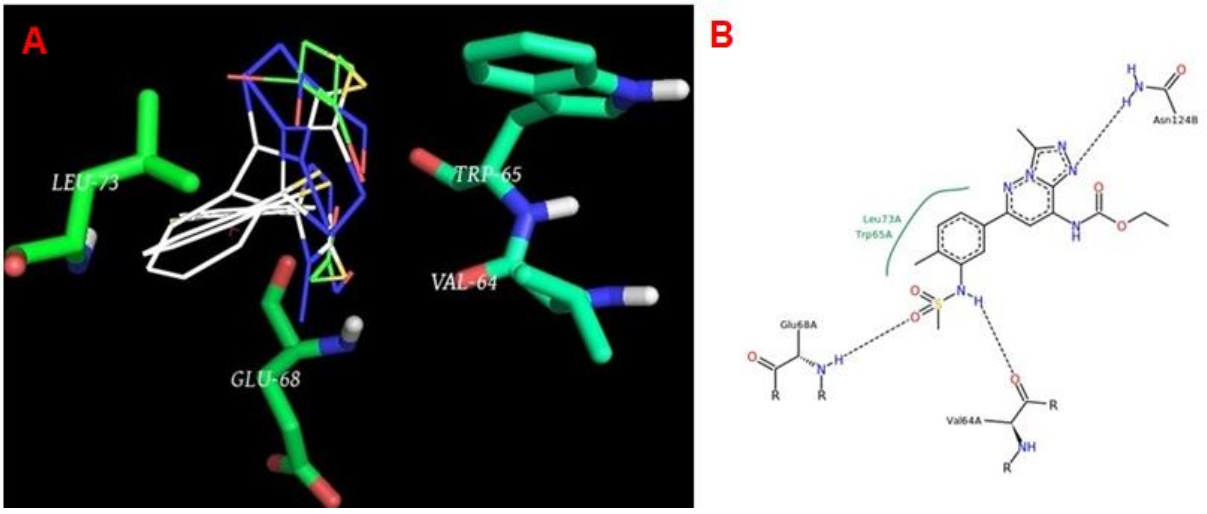


Fig 43. A; Cluster of conformers within the active site of the target protein of *Leishmania donovani* (pdb id: 5fea), **B;**Poseview of standard antileishmanial within the active site of 4kr4.

The binding mode of the highest active test compound exhibiting appreciable antileishmanial activity was investigated by docking studies. Protein domain of *Leishmania donovani* was obtained from protein repository. Compound 6e against, 2-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide, the best against *Leishmania donovani* was further considered for docking. This test compound was docked within the active site of 5FEA, domain protein of Leishmania strain. The active site residues were traced from the pose view as collected from rcsb (Figure 43A). The important residues like val64, glu68, leu73 were clearly found to encase few conformers of the test compound, 6e (Figure 43B).

8.3. Conclusion

A series of twelve 5-substituted-1,2,4-triazole linked hydroxamic acid derivatives 6a-6l were synthesized and evaluated for their in vitro antileishmanial efficacy and found to be potent to moderately active in inhibiting the pathogenic growth. The aryl group with its substituent leaves a mixed opinion with respect to the biological interaction. The electron donating ability of methyl, mercapto, amino group strengthen the binding affinity of the aryl group with the active

site of the target might be one of strong supporting evidence for their potency, but in the same ground how this nitro group being a strong electron group helps in lifting the chemotherapeutic index is difficult to establish. Therefore, the series requires to be well extended. Thus, it could be concluded that the synthesized 1,2,4-triazole linked hydroxamic acid derivatives with electron donating groups at 5-position of the 1,2,4-triazole moiety may provide a therapeutically effective chemical framework from which potential antileishmanial agents may be developed further.

Chapter 9

9. References

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Chapter 10

10. Future aspects

In this work I have prepared different types of 4-amino-5-((aryl substituted)-4h-1, 2, 4-triazole-3-yl) thio linked hydroxamic acid derivatives with the aim to evaluate their antibacterial, antifungal, antioxidant, anticancer and antileishmanial efficacy. Molecular mechanism of the therapeutic potency of the synthesized compounds still to investigate. Evaluation of the signaling pathways could be carried out in future to elicit the biological potency of the synthesized compounds. This chemical frame work could also lead to produce compound with more efficacy with less toxicity.

Chapter 11

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 3, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ANTIMICROBIAL INVESTIGATION AND BINDING MODE ANALYSIS OF SOME NEWLY SYNTHESIZED 4-AMINO-5-((ARYL SUBSTITUTED)-4H-1, 2, 4-TRIAZOLE-3-YL)-THIO LINKED HYDROXAMIC ACID DERIVATIVES

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Received: 22 November 2018, Revised and Accepted: 04 January 2018

ABSTRACT

Objective: A series of 5-substituted-4-amino-1, 2, 4-triazole-linked hydroxamic acid derivatives have been synthesized and explored *in vitro* to evaluate antibacterial and antifungal activities.

Methods: Different 5-phenyl group substituted-4-amino-1,2,4-triazole-3-thiol reacted with chlorine substituted hydroxamic acid to produce the desired compounds and characterized spectroscopically. Minimum inhibitory concentration (MIC), zone of inhibition (ZOI), growth kinetic studies, and scanning electron microscopy (SEM) were employed to elicit the antimicrobial efficacy of synthesized compounds against a wide range of bacterial and fungal strains.

Results: Compounds 6a, 6b, 6d, and 6k (MIC of 25 µg/ml) have been found to be more potent against *Klebsiella pneumoniae*, *Bacillus cereus, Bacillus pumilus*, *Micrococcus luteus,* and *Pseudomonas aeruginosa*, compounds 6a-6d, 6k, and 6l (MIC of 25–50 µg/ml) have shown potent antibacterial efficacy against *Klebsiella pneumonia*, *P. aeruginosa,* and *Vibrio cholera* compare to the standard drug amoxicillin (MIC of 60 µg/ml, 65 µg/ml, and 25 µg/ml, respectively). Screening for the antifungal activity revealed that the compounds were found to be most active against *Candida albicans* (6a, 6b, and 6l), *Candida tropicalis* (6b and 6d), and *Aspergillus niger* (6a, 6b, 6d, and 6j) with MIC of 15–25 µg/ml. Bacteriostatic and fungistatic effect of titled compounds was revealed from growth kinetics study.

Conclusion: Electron donating group at the 5-position of the 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives conferred the biological effectiveness of the synthesized compounds and also offer a therapeutically effective prototypical structure for further development of new chemical entities with superior antimicrobial activity.

Keywords: 1,2,4-triazole, Hydroxamic acid, Antibacterial, Antifungal.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i3.30882

INTRODUCTION

The microbes are getting resistant toward the existing chemotherapeutics in alarming rate which is not only a major concern for public health but also a challenge for the scientific community globally, and the number of cases of multidrug-resistant bacterial infections is increasing nowadays [1]. In clinical practice, the infections caused by the Gram-positive bacteria are very common. However, the infection caused by them is sometime severe. *Bacillus cereus*, *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Micrococcus luteus*, *Bacillus pumilus*, *Bacillus subtilis,* and *Enterococcus faecalis* are some important Gram-positive bacteria causing serious infection in the community, whereas *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium* are very common Gram-negative bacteria. Among them, *E. coli* is the most common one. The mortality is higher for infections caused by Gram-negative bacteria than the Gram-positive one. To overcome various microbial infections specifically fungal infection, a large number of triazole drugs have been successfully developed [2]. Azole compounds showed the effect by inhibiting lanosterol 14α-demethylase (CYP51) which catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis [3] resulting in accumulation of toxic methyl-sterols in membranes leading to fungistatic or fungicidal effect [4]. Some important azoles derivatives such as voriconazole, itraconazole, posaconazole, and fluconazole (Fluc) are used for the treatment of fast-growing fungal infections, which are the leading cause of mortality and morbidity in immunocompromised patients [5]. However, Fluc is not effective against aspergillosis due to its severe drug resistance [6,7].

These scenarios are highlighting the urgent need for novel, efficacious, less toxic, and safe drug candidates in the pipeline. The heterocyclic compounds containing nitrogen, sulfur, and oxygen have an enormous significance in the field of medicinal chemistry [8]. To address the concern, we develop a series of novel 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives. Assembling the different pharmacophores in a single frame always leads to compounds having fascinating biological profile [9]. Triazole is one of the pharmacophores in the synthesized compounds. Chemical compounds possess triazole as a core; always play a significant role in the field of medicinal chemistry. Like triazole, imidazole moiety has widely used to synthesize compounds to treat diseases causing by different microorganisms. Even though imidazole and triazole have a similar mode of action, triazoles have several advantages over imidazole, like, oral bioavailability, slow metabolic rate and have minimal effect on sterol synthesis in humans. For these, use of triazole moiety increases rather than the imidazole [10]. Triazole is well known for its antimicrobial property and widely used to treat microorganisms associated diseases [11]. Besides, the antibacterial [12-15] and antifungal [16-19] activity; 1,2,4-triazole possess, anti-inflammatory [20], as well as antioxidants properties [21]. Various well-known drug commercialized in the market possesses 1,2,4-triazole moiety such as Fluc, terconazole,

itraconazole, [22,23] triazolam [24], and ribavirin [25]. Different S and N-bridged heterocycles can be synthesized by 1,2,4-triazoles as it possesses nucleophilic centers. Triazolothiazines, triazolothiadiazoles, triazolothiazepines, triazolothiadiazines, and thiazolotriazoles can be synthesized from triazoles, having versatile therapeutic potentials. Another moiety in our synthetically designed compounds is hydroxamic acid. It is another interesting moiety possess antibacterial, antifungal [26,27], anti-inflammatory [28,29], antitumor [30], and anticancer agents [31]. Hydroxamic acid and its derivatives can exert versatile activity by inhibiting several enzymes, such as urease [32], matrix metalloproteinases [33,34], ribonucleotid reductase [35], and 5-lipoxygenase [36]. Hydroxamic acid has the ability to chelate with metals. Few ion exchange resins are built on hydroxamic acids [37]. A number of hydroxamic acid derivatives are used as chemotherapeutic agents such as ibuproxam, hydroxycarbamide, desferrioxamine B, oxametacin, adrafinil, and bufexamac [38]. Hence, considering the above fact we have designed and synthesized some novel 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives and evaluate their activity against several Gram-positive, Gram-negative bacteria, and a number of fungal strains.

METHODS

All the chemicals were procured from Sigma-Aldrich, India, Spectrochem, Merck India Pvt. Ltd., and the media were of purchased from HiMedia. Dichloromethane and dimethyl sulfoxide (DMSO) were procured from Merck, India; all other chemicals used were of analytical grade. In process monitoring of reaction was done on activated silica gel coated plates and the solvent system used was n-hexane:ethylacetate. The melting points of the synthesized compounds were measured by the capillary method and are reported uncorrected. The Fourier-transform infrared (FT-IR) spectra were measured in Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA), ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on AV300 Digital FT NMR Spectrometer, Bruker at 300 MHz using DMSO-d_6 as the solvent and tetramethylsilane as an internal standard, ^{13}C NMR spectra were obtained at 500 MHz (Bruker, Germany), DMSO-d_6 as the solvent. Mass spectra (m/z) of the compounds were recorded on JEOL-JMS 700 spectrometer using electron ionization technique. Scanning electron microscopy (SEM) study of the synthesized compounds was analyzed using SEM JEOL, Tokyo, Japan.

SYNTHETIC PROCEDURE

General procedure for the synthesis of first intermediate compound (1a-1f)

Substituted-aryl-acid was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. sulfuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with a small amount of activated anhydrous sodium sulfate. The ester in the form of oil was collected by means of distilling out the ether.

General procedure for the synthesis of Arylhydrazide (2a-2f)

Esterified compounds (1a-1f) (equimolar) were dissolved in 30 ml of ethanol, and hydrazine hydrate (equimolar) was added dropwise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation, and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried, and recrystallized from dehydrated ethanol to yield white crystals.

General procedure for the synthesis of potassium 2-*substituted*-hydrazine-1-carbodithioate (3a-3f)

Potassium hydroxide (equimolar) was dissolved in 75 ml of absolute alcohol, and arylhydrazide (2a-2f) (equimolar) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulfide was added (equimolar) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

General procedure for synthesis of 4-amino-5-(*substituted*-phenyl)-4H-1,2,4-triazole-3-thiol (4a-4f)

A suspension of potassium 2-*substituted* hydrazine-1-carbodithioate (3a-3f) in 20 ml of water and hydrazine hydrate was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added dropwise to make the reaction mixture strongly acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

General procedure for the synthesis of *substituted*-chloro-N-hydroxyacetamide (5a-5b)

0.01 mol of chloroacetylchloride (to synthesize 5a)/3-chloro propionylchloride (to synthesize 5b) was dissolved in 30 ml of methanol. To the reaction mixture, hydroxylamine stock solution (0.04 mol of hydroxylamine hydrochloride) was dissolved in 12 ml of hot methanol. The mixture was stirred for 5 min and to it, a solution of 0.06 mol potassium hydroxide in 10 ml of methanol was added dropwise. The resulting solution was cooled at room temperature and filtered to obtain 2 mol of stock solution was added dropwise and the mixture was stirred for 2 h. The precipitate thus obtained was washed with ether and recrystallized from methanol.

General procedure for the synthesis of title compounds (6a-6l)

To an equimolar mixture of 4-amino-5-(*substituted*-phenyl)-4H-1,2,4-triazole-3-thiol and *substituted*-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20 ml water was added and extracted with chloroform thrice; aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Antimicrobial activity

Microbial strains

In vitro antimicrobial activity was evaluated against 15 different pathogenic bacteria include both Gram-negative such as *S. typhi* 62, *K. pneumoniae* ATCC 10031, *Vibrio cholerae* VC 20, *P. aeruginosa* 25619, *Shigella sonnei* NK 4010, *E. coli* ATCC 25923, and *S. typhimurium* NTCC 74, and Gram-positive such as *Bacillus polymyxa* 4747, *B. cereus* 479, *Staphylococcus aureus* ATCC 29737, *E. faecalis* 28, *Bordetella bronchiseptica* 4617, *Micrococcus luteus* 10240, *B. pumilus* 148884, and *Bacillus subtilis* 6673, respectively, and seven different fungi such as *Candida albicans* MTCC 183, *Candida tropicalis* MTCC 2795, *Cryptococcus neoformans*, *Aspergillus niger* MTCC 281, *Microsporum gypseum*, *Penicillium chrysogenum*, and *Cladosporium sp*. All these microbial strains were collected from Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Preparation of inoculums

Nutrient broth was used to grow the bacterial strains at 37°C for 24 h whereas Czapek Dox media were used to culture the fungal strains at 25–30°C for 48–72 h. Afterward, both the bacterial and fungal strains were compared to 0.5 McFarland standards [39] and made the dilution with sterile normal saline was so that produced an initial suspension of 2×10^6 CFU/ml for further use.

Determination of minimum inhibitory concentration (MIC)

To determine the MIC of the synthesized compounds agar dilution method was employed as per the National Committee for Clinical Laboratory Standards (NCCLS) 2006 protocol [40]. The synthesized compounds were initially dissolved in a suitable solvent and then serial

dilution was made to obtain the concentrations 10, 25, 50, 75, 100, 150, 200, 250, 350, and 400 µg/ml. On the separate agar plates with different drug concentrations inocula (2×10^6 CFU/mL) were spotted and incubated for 24 h at 37°C (for bacterial strains). Amoxicillin (Amx) was used as a reference standard. Whereas, for fungal strains, unlike agar plates, agar media having different drug concentrations were placed as a slant in separate test tubes, and the fungal inocula (2×10^6 CFU/mL) were streaked and incubated further for 48–72 h at 28°C. Fluc was used as a reference standard. MIC was recorded for any lowest concentration where complete growth inhibition of microorganisms was observed visually.

Determination of zone of inhibition (ZOI)

The well-diffusion method was used to determine the ZOI. For that method, NCCLS 2004 guideline was followed. Briefly, agar plates were prepared, and 100µl of bacterial/fungal cell suspension (2×10^6CFU/mL) was spread on solid agar plates using a sterile spreader. Sterile borer was used to make the wells, and the tested compounds at their respective MIC was added to the wells and incubated for 24 h at 37°C (for bacteria) and 48–72 h at 28°C (for fungus). Fluc and Amx were used as a reference standard to compare antifungal and antibacterial activity, respectively, of the tested compounds. The zone diameters were measured, and the experiment was performed in triplicates.

Growth kinetic studies

Growth kinetics studies were carried out to those compounds which have shown higher ZOI at their respective MIC as well as for those have comparable and significant MIC values against the MIC of standard drugs for any bacterial and fungal strains used in the experimental design. Thus, selected tested compounds at their concentration of 2× MIC were exposed to Gram-positive and Gram-bacteria to carry out the study. For any given bacterial strain, 1 ml overnight cultured bacterial suspension was taken in two separate test tubes containing 4 ml fresh nutrient broth media and incubated for 2–3 h at 37°C. After that in one tube, tested compound was added and another tube was used as control (without treatment). At each predetermined time point, 100µl of bacterial suspension was taken from the tubes, diluted in sterile water to achieve the bacterial concentration of 2×10^6 CFU/mL. From that diluted suspension, 100µl was taken to spread on a solid agar plate and incubated for 24 h at 37°C. Afterward, a number of the colony was counted for both test and control [41].

For fungal strains, fungal spores at a concentration of 1×10^5 CFU/mL were inoculated 1 mL of Czapek Dox medium and incubated with or without tested compounds for 48 h with shaking (50 rpm) at 28°C. 100µl from each medium was taken to dilute in sterile water and spread onto Czapek Dox agar plate and incubated at 28°C for 48–72 h, numbers of the colony-forming unit were counted for test and control [42].

By calculating the viable counts at each time point, a 24 h time-kill curve was plotted by plotting log CFU/mL against time for bacterial strains and for fungal strains 96 h time-kill curve were plotted. The experiment was performed in triplicates and expressed as mean ± SD.

SEM

Each bacterial and fungal strain was inoculated in two sets, one was treated as test and another was control. After 2 h tested, compounds were inoculated in tube marked as test. After a sufficient incubation period, cultures were centrifuged at 3000 rpm for 10 min, and the supernatant was discarded to obtain cells pellet. Cells were resuspended in media specific for bacteria and fungus and kept for 10 min and again centrifuged at 3000 rpm for 10 min. Fixation of cells was done with 4% glutaraldehyde in 0.1 M phosphate buffer and kept for 2–3 h and again centrifuged at 3000 rpm for 10 min and the supernatant was discarded. Cells were washed with sterile water and subjected to dehydration by a series of alcohol washing with increasing strength of 30%, 50%, 70%, and 100%, respectively. A drop was put on clean glass slide and dried. Platinum coating was done and the sample was examined under SEM [43].

In silico target binding study

AutoDock Vina [44] using the Lamarckian genetic algorithm for the prediction of binding affinity and searching for the optimum binding site together with the AutoDock Tools (ADT) was employed to set up and perform blind docking calculations of the triazole derivatives binding to substrate binding site of respective enzymes. The membrane protein of *S. typhi* (protein data bank [PDB] id: 4KR4); chimeric CYP51 (PDB id: chimeric 1EA1); and crystal structure of cytochrome P450 14-alpha-sterol demethylase (Cyp51) from *Mycobacterium tuberculosis* in complex with azole inhibitors [45] were obtained from the PDB (www.rcsb.org/pdb) [46] at a resolution of 1.60 Å was constructed using AutoDock4 package to study the target binding interaction of all the compounds under study. The coordinates of tested compounds were taken from their two-dimensional structures sketched from ACD ChemSketch Freeware and converted to the PDB format using Open Babel [47].

The target (respective proteins) and the ligand (triazoles) files were prepared using ADT. The water molecules were deleted, and polar hydrogen atoms and Gasteiger charges were added to both the target molecule and the ligands. All other bonds were made rotatable. The target was saved in pdbqt format, and a configuration file was created. Later docking was run. AutoDock generated nine possible binding conformations for each ligand, and the root-mean-square cluster tolerance was set to 2.0 Å in each run. The binding mode and interactions were analyzed for the significant conformers of the compounds under study. All calculations were performed in a Dell system (3.4 GHz processor, 2GB RAM, 320 GB Hard disk operating system). For each of the docking cases, the lowest energy docked conformation, according to the AutoDock scoring function, was selected as the binding mode. Visualization of the docked pose was done using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC) molecular graphics program.

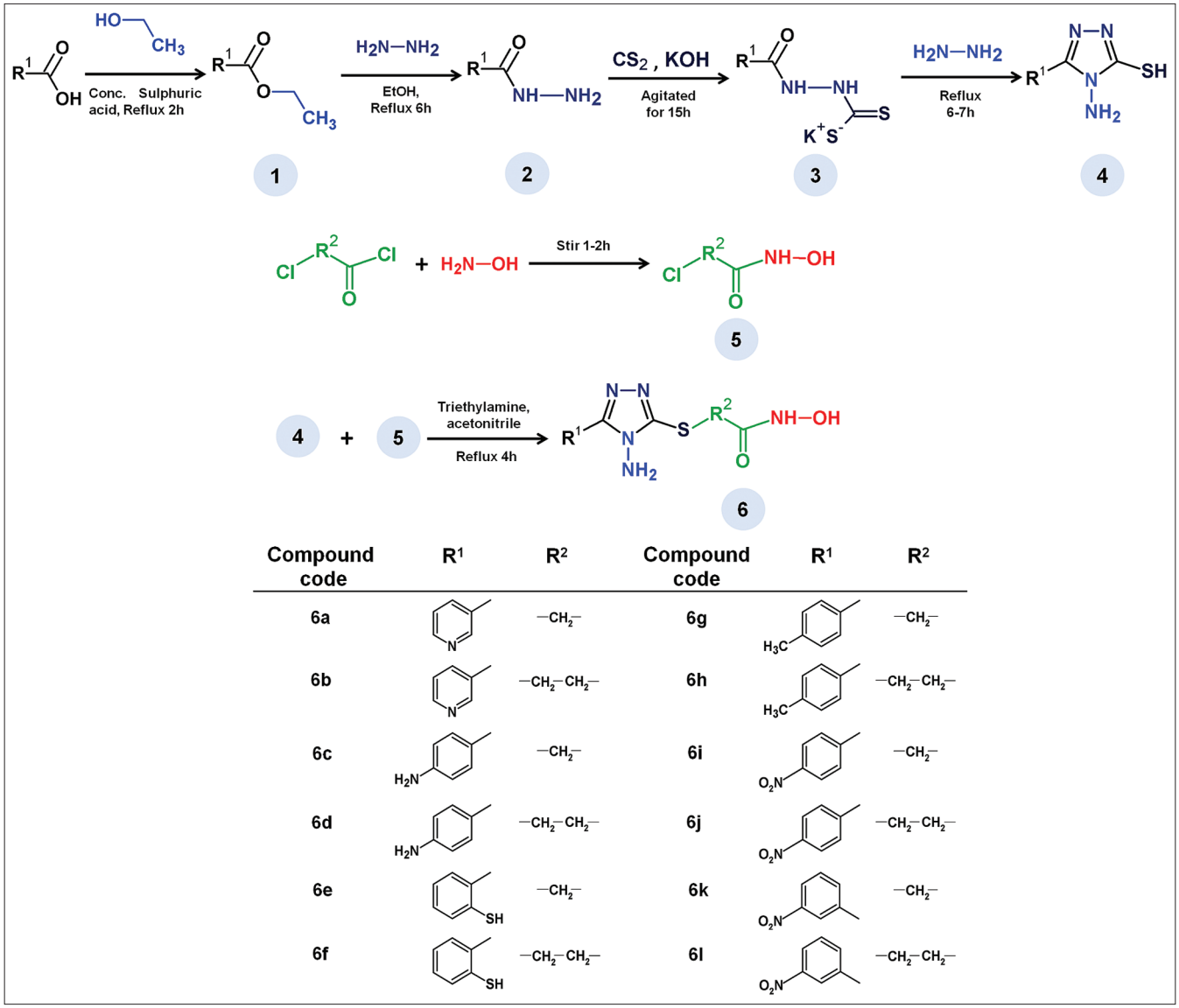
Docking was processed with setting of the grid sizes for 4KR4; 30, 26, and 24 and for chimeric 1EA1 40, 34, and 24 along the X-, Y-, and Z-axes with 1Å spacing which covered all the active residues. In a similar way, the grid center of each target was set for 4KR4; 34.52, 21.86, and 44.96 and for chimeric 1EA1 12.04, 2.87, and 62.50 Å for the protein.

Statistical analysis

Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. $p<0.01$ was considered as statistically significant whereas $p>0.05$ was considered to be statistically not significant.

RESULTS AND DISCUSSION

The synthetic route of the proposed scaffold was depicted in Scheme 1. A series of novel 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide and 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide(6a-6l) compounds were synthesized by reacting 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol with the respective 2-chloro-N-hydroxy acetamides/3-chloro-N-hydroxypropanamide (Scheme 1). Briefly, the reaction was carried out in three parts, i.e., synthesis of 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol and synthesis of 2-chloro-N-hydroxy acetamides/3-chloro-N-hydroxypropanamide then both were coupled to synthesis of the final compounds. 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol were synthesized by Reid-Heindal method [48], i.e., aryl acids were taken as a starting material and refluxed for 2 h with ethanol and concentrated sulfuric acid to produce the corresponding aryl esters. The aryl esters were further refluxed with hydrazine hydrate to yield corresponding aryl hydrazides. Then, in the presence of ethanolic potassium hydroxide, the aryl hydrazides were condensed with carbon-di-sulfide to produce potassium-3-aroyldithiocarbazates. These aroyldithiocarbazates were cyclized to produce corresponding aryl substituted 1,2,4-triazole. In another part, chloroacetylchloride or chloropropionylchloride reacted with hydroxyl amine hydrochloride to yield 2-chloro-N-hydroxy



Compound code	R^1	R^2	Compound code	R^1	R^2
6a		$-CH_2-$	6g		$-CH_2-$
6b		$-CH_2-CH_2-$	6h		$-CH_2-CH_2-$
6c		$-CH_2-$	6i		$-CH_2-$
6d		$-CH_2-CH_2-$	6j		$-CH_2-CH_2-$
6e		$-CH_2-$	6k		$-CH_2-$
6f		$-CH_2-CH_2-$	6l		$-CH_2-CH_2-$

Scheme 1: Synthetic route for target compounds 6a–6l

acetamides or 3-chloro-N-hydroxypropanamide, respectively. In the final step, 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxy acetamides or 3-chloro-N-hydroxypropanamide were condensed in the presence of triethylamine to produce the final compound, i.e., 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide or 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide, respectively. All the final compounds (6a-6l) were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and mass spectral data.

2-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6a)

Yield: 1.2 g (70%), mp: 200–202°C, FTIR (KBR) (cm^{-1}): 3420.39 (N-H stretching), 3028.37 (aromatic C-H stretching), 2981.27 (aliphatic C-H stretching), 1431.09 (aromatic C=C stretching), 1618.65 (C=O stretching), 1530.36 (C=N stretching), 1303.91 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0370 (s; NH), 9.1331 (s; OH), 8.3740–7.5855 (m; Ar-H), 5.7858 (s; NH_2), 1.0373 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4713 (CH), 147.5985 (CH), 124.2174 (CH), 133.6890 (C), 148.3817 (C), 30.6083 (CH2), 167.2465 (C). Mass: [EI+] ($C_9H_{10}N_6O_2S$) 266 Da; calculated for $C_9H_{10}N_6O_2S$, C, 40.60; H, 3.79; N, 31.56; O, 12.02; S, 12.04 found: C, 40.72; H, 3.73; N, 31.60; O, 11.96; S, 11.98.

3-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6b)

Yield: 1 g (73%), mp: 197–200°C, FTIR (KBR) (cm^{-1}): 3412.17 (N-H stretching), 3026.40 (aromatic C-H stretching), 2930.53 (aliphatic C-H stretching), 1431.34 (aromatic C=C stretching), 1618.84 (C=O stretching), 1530.61 (C=N stretching), 1304.29 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0378 (s; NH), 9.1305 (s; OH), 8.3704–7.5807 (m; Ar-H), 5.7834 (s; NH_2), 1.1560–1.0098 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4718 (CH), 147.5995 (CH), 124.2179 (CH), 133.6898 (C), 148.3825 (C), 30.6088 (CH_2), 25.7294 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 found: C, 42.92; H, 4.36; N, 29.93; O, 11.39; S, 11.4.

2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6c)

Yield: 720 mg (768%), mp: 212–215°C, FTIR (KBR) (cm^{-1}): 3372.34 (N-H stretching), 3038.50 (aromatic C-H stretching), 2918.46 (aliphatic C-H stretching), 1499.91 (aromatic C=C stretching), 1693.64 (C=O stretching), 1536.51 (C=N stretching), 1334.46 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.80011 (s; NH), 9.32056 (s; OH), 7.62590–6.52428 (m; Ar- H), 5.91882 (s; NH_2), 1.24555 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8490 (CH), 120.9804

(CH), 128.4892 (CH), 147.9976 (C), 148.7785 (C), 30.6102 (CH_2), 167.2496 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 found: C, 42.53; H, 4.42; N, 29.82; O, 11.26; S, 11.57.

3-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6d)

Yield: 850 mg (71%), mp: 210–214°C, FTIR (KBR) (cm^{-1}): 3341.13 (N-H stretching), 3054.69 (aromatic C-H stretching), 2899.90 (aliphatic C-H stretching), 1442.34 (aromatic C=C stretching), 1678.97 (C=O stretching), 1574.30 (C=N stretching), 1271.78 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.7077 (s; NH), 9.4001 (s; OH), 7.7603–7.2099 (m; Ar- H), 5.2626 (s; NH_2), 1.2561 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8498 (CH), 128.4889 (CH), 120.9809 (C), 147.9981 (C), 148.7789 (C), 30.6109 (CH_2), 25.3169 (CH_2), 167.2498 (C). Mass: [EI+] ($C_{11}H_{14}N_6O_2S$) calc. 294.09 Da, Found: 294 Da; calculated for $C_{11}H_{14}N_6O_2S$, C, 44.89; H, 4.79; N, 28.55; O, 10.87; S, 10.89 found: C, 45.05; H, 4.36; N, 28.76; O, 11.09; S, 10.74.

2-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6e)

Yield: 1.12g (70%), mp: 105–109°C, FTIR (KBR) (cm^{-1}): 3367.57 (N-H stretching), 3082.03 (aromatic C-H stretching), 2980.41 (aliphatic C-H stretching), 1458.46 (aromatic C=C stretching), 1696.7 (C=O stretching), 1563.15 (C=N stretching), 1287.18 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.98857 (s; NH), 9.58518 (s; OH), 8.04105–7.36677 (m; Ar- H), 5.31007 (s; NH_2), 1.36065 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1776 (CH), 126.1872 (CH), 127.2028 (CH), 131.2604 (C), 133.5519 (C), 138.5647 (CH), 148.3655 (C), 30.6009 (CH_2), 165.5395 (C). Mass: [EI+] ($C_{10}H_{11}N_5O_2S_2$) calc. 297.04Da, Found: 297 Da; calculated for $C_{10}H_{11}N_5O_2S_2$, C, 40.39; H, 3.73; N, 23.55; O, 10.76; S, 21.57 found: C, 40.45; H, 4.34; N, 29.94; O, 11.21; S, 11.52.

3-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6f)

Yield: 1.3 g (74%), mp: 100–103°C, FTIR (KBR) (cm^{-1}): 3385.76 (N-H stretching), 3093.54 (aromatic C-H stretching), 2980.07 (aliphatic C-H stretching), 1458.33 (aromatic C=C stretching), 1695.47 (C=O stretching), 1562.97 (C=N stretching), 1286.84 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.00011 (s; NH), 9.51002 (s; OH), 8.03405–7.33288 (m; Ar- H), 5.30011 (s; NH_2), 1.37415–1.32802 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1786 (CH), 126.1852 (CH), 127.2035 (CH), 131.2606 (C), 133.5505 (C), 138.5682 (CH), 148.4652 (C), 30.6089 (CH_2), 25.8079 (CH_2), 165.5396 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S_2$) calc. 311.05Da, Found: 311 Da; calculated for $C_{11}H_{13}N_5O_2S_2$, C, 42.43; H, 4.21; N, 22.49; O, 10.28; S, 20.60 found: C, 41.99; H, 4.28; N, 30.06; O, 11.39; S, 11.72.

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6g)

Yield: 1.8 g (80%), mp: 138–140°C, FTIR (KBR) (cm^{-1}): 3343.96 (N-H stretching), 3048.17 (aromatic C-H stretching), 2917.92 (aliphatic C-H stretching), 1443.74 (aromatic C=C stretching), 1667.23 (C=O stretching), 1514.60 (C=N stretching), 1269.08 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.64368 (s; NH), 9.30012 (s; OH), 7.82233–7.25141 (m; Ar- H), 5.60085 (s; NH_2), 1.13647 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 129.0487 (CH), 129.2014 (CH), 129.2701 (CH), 127.9722 (C), 142.9518 (C), 129.3370 (CH), 148.1818 (C), 30.4883 (CH_2), 21.0502 (CH_2), 167.2505 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S$) calc. 279.08 Da, found: 279 Da; calculated for $C_{11}H_{13}N_5O_2S$, C, 47.30; H, 4.69; N, 25.07; O, 11.46; S, 11.48 found: C, 48.10; H, 4.18; N, 29.86; O, 11.14; S, 11.57.

3-((4-amino-5-(p-tolyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6h)

Yield: 1.4 g (73%), mp: 148–150°C, FTIR (KBR) (cm^{-1}): 3342.98 (N-H stretching), 3071.72 (aromatic C-H stretching), 2918.91 (aliphatic C-H stretching), 1444.07 (aromatic C=C stretching), 1667.44 (C=O stretching), 1515.06 (C=N stretching), 1281.37 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.67881 (s; NH), 9.37850 (s; OH), 7.82335–7.25908 (m; Ar- H), 5.59985 (s; NH_2), 1.16979–1.12207 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 129.0512 (CH), 129.2045 (CH), 129.2723 (CH), 127.9750 (C), 142.9543 (C), 129.3390 (CH), 148.1827 (C), 30.4859 (CH_2), 25.8094 (CH_2), 21.0517 (CH_3), 167.2530 (C). Mass: [EI+] ($C_{12}H_{15}N_5O_2S$) calc. 293.09 Da, found: 293 Da; calculated for $C_{12}H_{15}N_5O_2S$, C, 49.13; H, 5.15; N, 23.87; O, 10.91; S, 10.93 found: C, 48.98; H, 5.10; N, 29.95; O, 10.97; S, 10.99.

Table 1: MIC values of synthesized compounds (6a-6l)

Organisms	**MIC (µg/ml)**												
Bacterial strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
Salmonella typhi	25	25	50	50	-	-	-	-	-	-	50	100	10
Klebsiella pneumoniae	25	50	25	25	75	100	-	-	-	-	-	-	60
Bacillus polymyxa	100	100	-	-	50	50	-	-	-	-	100	100	32
Bacillus cereus	50	50	50	25	-	-	-	-	50	75	75	50	12
Vibrio cholerae	50	75	-	-	-	-	75	100	-	-	50	25	25
Staphylococcus aureus	100	150	100	50	-	-	-	-	-	-	-	-	16
Enterococcus faecalis	-	-	-	-	-	-	-	-	200	200	150	100	25
Bordetella bronchiseptica	50	50	-	-	50	50	-	-	50	100	-	-	25
Micrococcus luteus	-	-	-	-	-	-	100	150	-	-	25	25	0.5
Pseudomonas aeruginosa	25	25	50	50	-	-	-	-	-	-	25	25	65
Shigella sonnei	100	100	-	-	-	-	-	-	-	-	-	-	32
Escherichia coli	-	-	100	100	-	-	100	100	-	-	100	100	10
Bacillus pumilus	25	25	50	25	-	-	-	-	50	50	50	75	2
Salmonella typhimurium	-	-	100	200	200	200	-	-	-	-	150	100	25
Bacillus subtilis	25	25	25	50	-	-	100	100	-	-	100	150	8
Fungal stains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Fluc**
Candida albicans	15	25	50	25	-	-	100	50	50	100	50	20	12.5
Candida tropicalis	50	20	100	20	-	-	200	100	-	-	-	-	10
Candida neoformans	50	50	-	-	-	-	100	200	200	50	50	50	15
Aspergillus niger	20	20	50	25	300	200	100	50	50	25	50	100	18
Microsporum gypseum	-	-	300	200	-	-	500	350	500	300	50	50	16
Penicillium chrysogenum	-	-	300	250	-	-	400	400	-	-	-	-	56
Cladosporium	250	300	-	-	-	-	400	200	250	300	-	-	32

Amx: Amoxicillin, Fluc: Fluconazole

Table 2: ZOI of tested compounds (6a-6l)

Organisms	Zone of Inhibition (mm)												
Bacterial strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
Salmonella typhi	18.5±0.2	16.5±0.5	15.7±0.5	15.1±0.2	_	_	_	_	_	_	15.1±0.2	11.2±=0.1	21.0±0.9
Klebsiella pneumoniae	16.0±0.4	11.5±0.3	9.3±0.3	13.8±0.1	11.2±0.4	10.1±0.4	_	_	_	_	_	_	13.5±0.9
Bacillus polymyxa	13.3±0.3	16.4±0.4	_	_	15.4±0.1	12.2±0.1	_	_	_	_	11.5±0.4	10.6±0.2	18.7±0.2
Bacillus cereus	14.0±0.1	12.1±0.2	13.6±0.2	15.5±0.1	_	_	_	_	15.2±0.2	13.5±0.4	11.7±0.4	13.2±0.1	17.3±0.2
Vibrio cholerae	11.0±0.9	12.0±0.1	_	_	_	_	14.1±0.2	12.6±0.4	_	_	11.4±0.4	13.6±0.2	13.5±0.5
Staphylococcus aureus	15.3±0.2	12.5±0.4	12.0±0.6	14.7±0.2	_	_	_	_	_	_	_	_	16.5±0.9
Enterococcus faecalis	_	_	_	_	_	_	_	_	11.0±0.6	10.2±0.2	11.2±0.4	11.1±0.2	15.2±0.4
Bordetella bronchiseptica	10.4±0.4	10.5±0.5	_	_	11.1±0.2	10.0±0.1	_	_	11.0±0.6	12.9±0.2	_	_	15.3±0.2
Micrococcus luteus	_	_	_	_	_	_	14.2±0.1	12.9±0.4	_	_	11.4±0.4	9.5±0.2	18.7±0.6
Pseudomonas aeruginosa	14.1±0.2	13.6±0.2	13.1±0.3	11.5±0.3	_	_	_	_	_	_	13.5±0.4	12.6±0.2	16.9±0.2
Shigella sonnei	10.0±0.3	9.6±0.3	_	_	_	_	_	_	_	_	12.0±0.3	11.2±0.2	15.9±0.3
Escherichia coli	_	_	10.8±0.3	10.1±0.2	_	_	10.7±0.3	10.3±0.3	_	_	10.7±0.1	10.1±0.2	17.3±0.2
Bacillus pumilus	13.3±0.3	11.5±0.3	12.0±0.3	11.3±0.3	_	_	_	_	9.4±0.2	9.1±0.4	11.9±0.1	11.2±0.3	14.4±0.1
Salmonella typhimurium	_	_	11.0±0.1	10.1±0.2	12.1±0.2	11.1±0.3	_	_	_	_	13.7±0.2	12.5±0.3	16.9±0.2
Bacillus subtilis	13.3±0.2	11.4±0.2	12.1±0.3	11.1±0.2	_	_	12.4±0.2	11.9±0.3	_	_	12.8±0.2	11.8±0.3	13.9±0.3
Fungal strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Fluc**
Candida albicans	11.9±0.7	10.2±0.6	10.8±0.8	7.8 ±0.7	_	_	7.9 ±0.5	9.6 ±0.8	7.6 ±0.8	11.0±0.3	12.2±0.3	11.1±0.3	16.0±0.9
Candida tropicalis	9.4 ±0.8	9.2 ±0.9	8.0 ±0.6	9.2 ±0.8	_	_	9.5 ±0.7	9.4 ±1.2	_	_	_	_	16.3±0.4
Candida neoformans	7.3 ±0.5	8.4 ±0.6	_	_	_	_	8.7 ±0.6	7.5 ±0.7	8.4 ±0.7	10.3±0.3	12.9±0.3	12.6±0.3	14.2±0.6
Aspergillus niger	11.2 ±0.6	11.6 ±0.9	10.2 ±0.6	9.8 ±0.7	11.3±0.9	8.9±0.7	9.6 ±0.7	11.3 ±0.7	7.43 ±0.6	9.8 ±0.1	14.1±0.1	9.9 ±0.1	17.6±0.3
Microsporum gypseum	_	_	7.6 ±0.7	6.8 ±0.7	_	_	6.8 ±0.8	8.7 ±0.7	6.8 ±0.7	10.7±0.4	11.9±0.4	11.1±0.3	16.4±0.9
Penicillium chrysogenum	_	_	6.6 ±0.8	8.5 ±0.8	_	_	7.7 ±0.6	7.3 ±0.5	_	_	_	_	16.3±0.6
Cladosporium	7.5 ±0.6	7.2 ±1.0	_	_	_	_	5.8 ±0.6	8.3 ±0.9	6.5 ±0.8	10.1±0.4	_	_	15.2±0.9

Amx: Amoxicillin, Fluc: Fluconazole, experimental data expressed as mean±SD, n=3, SD: Standard deviation, ZOI: Zone of inhibition

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6i)

Yield: 1.0 g (67%), mp: 223–225°C, FTIR (KBR) (cm^{-1}): 3351.84 (N-H stretching), 3070.84 (aromatic C-H stretching), 2939.00 (aliphatic C-H stretching), 1451.03 (aromatic C=C stretching), 1698.61 (C=O stretching), 1572.72 (C=N stretching), 1288.70 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0948 (s; NH), 9.48836 (s; OH), 8.31648–7.97074 (m; Ar- H), 5.49898 (s; NH_2), 1.19998 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0874 (CH), 128.4884 (CH), 136.8090 (C), 147.9985 (C), 148.7815 (C), 30.6092 (CH_2), 167.2478 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, Found: 310 Da; calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 found: C, 39.76; H, 2.95; N, 26.73; O, 20.48; S, 10.13

3-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6j)

Yield: 1.2 g (71%), mp: 212–215°C, FTIR (KBR) (cm^{-1}): 3365.73 (N-H stretching), 3076.11 (aromatic C-H stretching), 2857.26 (aliphatic C-H stretching), 1475.39 (aromatic C=C stretching), 1681.59 (C=O stretching), 1519.24 (C=N stretching), 1311.25 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1975 (s; NH), 9.15008 (s; OH), 8.32100–7.98191 (m; Ar- H), 5.48758 (s; NH_2), 1.20831–1.16040 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0878 (CH), 128.4887 (CH), 136.8098 (C), 147.9989 (C), 148.7818 (C), 30.6095 (CH_2), 25.8370 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, found: 324 Da; calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 found: C, 41.14; H, 3.58; N, 26.06; O, 19.95; S, 10.07.

2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6k)

Yield: 1g (70%), mp: 220–224°C, FTIR (KBR) (cm^{-1}): 3308.80 (N-H stretching), 3082.67 (aromatic C-H stretching), 2926.24 (aliphatic C-H stretching), 1481.03 (aromatic C=C stretching), 1700.90 (C=O stretching), 1518.48 (C=N stretching), 1296.15 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1101 (s; NH), 9.4886 (s; OH), 8.9514–7.8044 (m; Ar- H), 5.8517 (s; NH_2), 1.2327 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0683 (CH), 131.2639 (CH), 132.0087 (C), 133.0016 (CH), 148.0011 (C), 148.9088 (C), 31.8313 (CH_2), 165.7338 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, found: 310 Da; calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 found: C, 38.80; H, 3.29; N, 26.98; O, 20.61; S, 10.30.

3-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6l)

Yield: 1.1 g (70%), mp: 210–212°C, FTIR (KBR) (cm^{-1}):. ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1199 (s; NH), 9.4883 (s; OH), 8.9488–7.1773 (m; Ar- H), 5.8455 (s; NH_2), 2.2546 (s; CH_2), 1.2186 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0678 (CH), 131.2635 (CH), 132.0088 (C), 133.0010 (CH), 148.0003 (C), 148.9085 (C), 31.8309 (CH_2), 25.9689 (CH2), 165.7335 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, found: 324 Da; calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 found: C, 40.64; H, 3.67; N, 25.99; O, 19.79; S, 9.92.

The synthesized compounds (6a-6l) were exposed to 15 different bacterial strains including both Gram-negative and Gram-positive bacteria and found to be efficacious at a range of MIC values between 25 and 200 µg/ml. Amx was used as a reference standard to compare the antibacterial activity of the synthesized compounds. The comparative MIC values of the compounds were tabulated in Table 1. The results showed that majority of the synthesized compounds elicited variable degrees of inhibition against the tested microorganisms. Compounds 6a, 6b, 6c, 6d, 6k, and 6l exhibited potent antibacterial activity against *K. pneumonia*, *B. cereus, P. aeruginosa, B. pumilus, S. typhi, B. subtilis,* and *V. cholerae* at a MIC of 25 µg/ml whereas compound 6e (75 µg/ml) has shown moderate activity compared to the standard having the MIC of 60 µg/ml. Compound 6f (100 µg/ml) has poor efficacy against the same strain where other compounds have shown no activity. Against *P. aeruginosa*, compounds 6a- 6d, 6k, and 6l (MIC of 25–50 µg/ml) have found to be more potent than standard compound (65 µg/ml) where other compounds have found to be no activity. Against the other bacterial strain compounds have shown moderate to less activity compared to the standard like, compound 6e, 6f (50 µg/ml) against *B. polymyxa*; compounds 6a, 6k, and 6l (50 µg/ml) against *V. cholerae*; compounds 6a, 6b, 6e, 6f, and 6i (50 µg/ml) against *B. bronchiseptica* were moderately active where other compounds have mild bioactivity against the tested bacterial strains, however, compared to the standard they have very less to no activity.

In vitro, antifungal activity was evaluated against seven different fungal strains and the result showed that the synthesized compounds were active against all tested fungi to some extent (Table 1). Among the tested compounds, compound 6a showed potent activity against *Candida albicans* at MIC 15 µg/ml which is nearer to standard Fluc (12.5 µg/ml), whereas compound 6l (20 µg/ml) was found to be moderately active against *Candida albicans*. Against *A. niger* compounds 6a and 6b (20 µg/ml) were potent whereas compounds 6d and 6j (25 µg/ml) were moderately active compared to the standard (18 µg/ml). On the other hand, compounds 6b and 6d (20 µg/ml) elicited moderate activity against *Candida tropicalis*.

The ZOI of the compounds which showed inhibitory efficacy against individual strains of bacteria and fungus, was calculated at their respective MIC and tabulated in Table 2 like, against *S. typhi* compounds 6a and 6b showed the ZOI of 18.5±0.2 mm (at MIC of 25 µg/ml) and 16.5±0.5 mm (at MIC of 25 µg/ml), respectively. Against *K. pneumoniae* compounds 6a, 6c, and 6d showed ZOI of 16.0±0.4 mm, 9.3±0.3 mm, and 13.8±0.1 mm (at MIC of 25 µg/ml), respectively. Compound 6e showed ZOI of 15.4±0.1mm (MIC = 50 µg/ml) against *B. polymyxa*. Compound 6l have a ZOI of 13.6±0.2 mm (MIC = 25 µg/ml) against *V. cholerae*. ZOI of 14.7±0.2 mm (MIC = 50 µg/ml) for compound 6d against *S. aureus*. 11.4±0.4 mm and 9.5±0.2 mm (MIC = 25 µg/ml) for compounds 6k and 6l against *M. luteus*. For compounds 6a, 6b, 6k, and 6l, ZOI was almost 13mm against *P. aeruginosa* at MIC of 25 µg/ml. ZOI of 13.3±0.3 mm and 13.3±0.2 mm were higher for compound 6a against *B. pumilus* and *B. subtilis*, respectively, at MIC of 25 µg/ml.

For fungal strains, it was observed that compound 6a has the ZOI of 11.9±0.7 mm (MIC = 15 µg/ml) for *C. albicans*; compound 6b showed 9.2±0.9 mm (MIC = 20µg/ml) for *C. tropicalis,* and against *A. niger* compounds 6a and 6b have shown almost similar ZOI of 11.2 ±0.6mm and 11.6 ±0.9 mm, respectively, at the MIC of 20µg/ml.

Compounds 6a, 6b, and 6l were most active against the fungal strain *C. albicans*. It was found very interesting that all of the synthesized compounds were active against *A. niger* even compounds 6e and 6f which were not effective against the any of the fungal strain (between the tested concentration to determine MIC) used for this experimental design, were active against *A. niger* although the MIC values were high enough, i.e., 300µg/ml and 200µg/ml, respectively, compared to the standard drug Fluc (18 µg/ml). However, compounds 6b and 6a showed potent antifungal activity against *A. niger* with ZOI of 11.6 ±0.9 mm and 11.2 ±0.6 mm at the MIC of 20 µg/ml.

SEM was carried out to assess morphological changes in bacterial cells following the exposure of most active compounds against selective bacterial as well as fungal strains; like, for bacterial strains, *B. pumilus* was treated with compound 6a and *P. aeruginosa* was treated with compound 6b whereas for fungal strains, *C. tropicalis* was treated with compound 6b and *C. albicans* was treated with compound 6a. The SEM analysis showed clear morphological changes in bacterial cells when treated with tested compounds. Fig. 1k and l depict the SEM image of control and treated *B. pumilus*, respectively. SEM micrographs of *B. pumilus* revealed that when the bacterium was exposed to the tested compound, the cells disrupted following alterations in the cell integrity. *P. aeruginosa* when treated with the tested compound (Fig. 1n), appeared to swell with loss of its normal morphology as depicted by the control (Fig. 1m). Moreover, based on the pathogenic mechanisms of *B. pumilus* and *P. aeruginosa*, an alternative strategy for the treatment

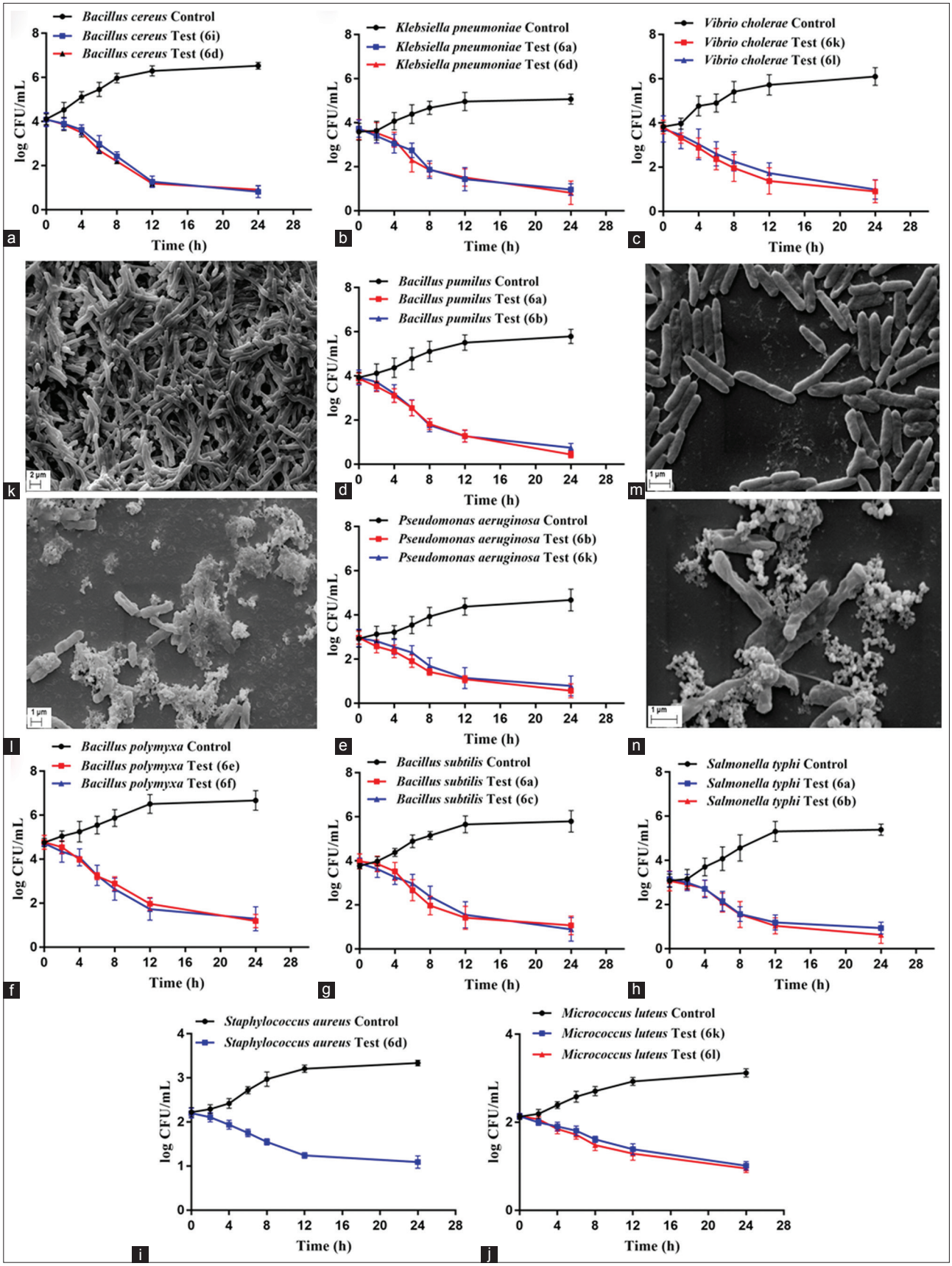


Fig. 1: Time-dependent *in vitro* growth curve of *Bacillus cereus* (a), *Klebsiella pneumoniae* (b), *Vibrio cholerae* (c), *Bacillus pumilus* (d), *Pseudomonas aeruginosa* (e), *Bacillus polymyxa* (f), *Bacillus subtilis* (g), *Salmonella typhi* (h), *Staphylococcus aureus* (i), *Micrococcus luteus* (j) at their 2×MIC values against test compounds and scanning electron micrograph of *B. pumilus* (k) before treatment, (l) after treatment; *P. aeruginosa* (m) before treatment, (n) after treatment

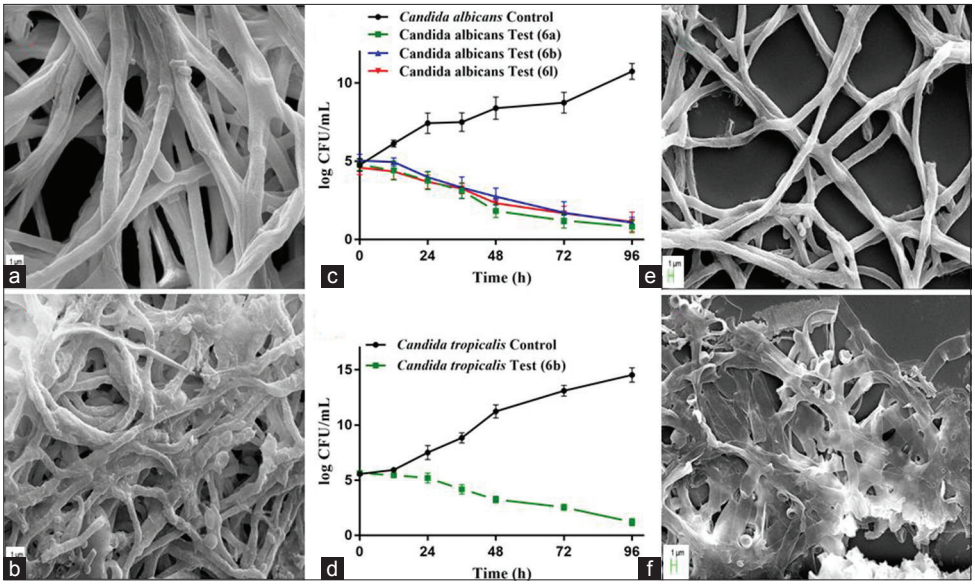


Fig. 2: Scanning electron micrograph of *Candida tropicalis* (a) before treatment, (b) after treatment*; Candida albicans* (e) before treatment, (f) after treatment and time-dependent *in vitro* growth curve of *C. albicans* (c) and *C. tropicalis* (d) at their ×2 MIC values against test compounds

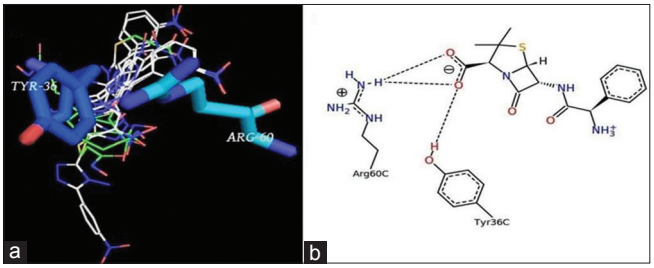


Fig. 3: (a) Cluster of conformers within the active site of the target protein of *Salmonella typhi* (pdb id: 4kr4), (b) PoseView of standard ampicillin within the active site of 4kr4

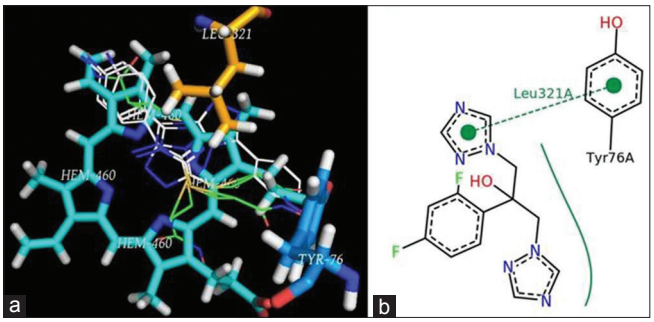


Fig. 4: (a) Cluster of conformers within the active site of the target enzyme of *Candida albicans* (pdb id: Chimeric 1ea1), (b) PoseView of standard fluconazole within the active site of 4kr4

of the infections caused by these organisms is of supreme importance in the current scenario.

Fig. 2a and b are the SEM images of control and treated *Candida tropicalis*, respectively, whereas, Fig. 2e and f show the SEM images of control and treated *Candida tropicalis*, respectively. In both the cases, structural changes in the SEM studies appeared progressively due to cohesion, diffusibility, agglomeration, and ultimate structure distortion and disintegration.

Growth kinetic study was done against the bacterial strains such as *B. cereus*, *K. pneumoniae*, *V. cholerae*, *B. pumilus*, *P. aeruginosa*, *B. polymyxa*, *B. subtilis*, *S. typhi*, *S. aureus,* and *M. luteus* for the tested compounds which showed higher ZOI at their respective MIC (Fig. 1a-J, respectively). Whereas, for fugal strains, compounds 6a, 6b, and 6l were studied for the growth kinetics evaluation against *C. albicans* and compound 6b for *C. tropicalis* displayed in Fig. 2c and d, respectively. Although almost all synthesized compounds have shown antifungal activity against *A. niger*, the growth kinetic study could not be performed as the *A. niger* strains forming very dark brown spores which could not be counted visually. From the growth kinetics data of bacteria as well as fungus from Figs. 1 and 2, respectively, it could be observed that the all best active compounds showed bacteriostatic and fungistatic activity.

It could be observed from the antimicrobial data that synthesized aryl substituted 1,2,4- triazole-linked hydroxamic acid derivatives showed moderate to high activity against tested bacterial as well as fungal strains. The antifungal enzyme, lanosterol-14α-demethylase is one of the primary targets of antifungal drug discovery. The molecular mechanism of triazole as an antifungal has already been established. All the compounds considered for the study possessed 1,2,4-triazole ring in it; therefore, all the compounds exhibited affinity toward the enzyme. The intensity of binding was high in compounds 6a, 6b, and 6l, which may be due to the ring nitrogen and the electronegative nitro group present in the aryl group attached to the central triazole, respectively. Triazole is well known for its antimicrobial activity. However, electron-rich aryl substitutions at 5- position of the 1,2,4-triazole could be an added benefit along with the linked hydroxamic acid. For many decades, the antimicrobial activity of different hydroxamic acid derivatives was evaluated. It was reported that hydroxamic acid is potent as well as a specific inhibitor of bacterial urease [49]. Hence, the antimicrobial activity of the synthesized compounds might be due to the partial effect of hindered urea metabolism by the hydroxamic acid part. It was also reported that the presence of -CONHOH group is very much necessary for the inhibition of microbial urease. In addition, alkyl group present in the hydroxamic acid part of the synthesized compounds influenced the effectiveness.

The binding mode of the highest active test compound exhibiting appreciable antimicrobial activity was investigated by docking studies. Crystal structure of *Salmonella typhi* was obtained from protein repository. On the other hand, no experimental structural information on the active site of the target enzyme *Candida* P450DM is available. It is observed from an extensive literature search that high homology exists

between the mycobacterium P450DM and *Candida* P450DM. Following the method of Rossello *et al.* [50], the chimeric enzyme for the *Candida albicans* (CACYP51) was developed from that of mycobacterium P450DM (MT CYP51) extracted from the PDB (entry code 1EA1). The residues that were arranged in a range of 7Å from Fluc were substituted with those of Candida P450DM. Substitutions were made by replacement of the residues Pro77, Phe78, Met79, Arg96, Met99, Leu100, Phe255, Ala256, His258, Ile322, Ile323, and Leu324 by Lys77, His78, Leu79, Leu96, Lys99, Phe100, Met255, Gly256, Gln258, His322, Ser323, and Ile324, which were thought to be necessary for the ligand-receptor interaction. Compound 6k, 2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide was within the active site of 4kr4. The active site composition was procured from rcsb and the binding pattern of ampicillin in Fig. 3b was set as standard. Almost all the docked conformers occupied the proximal vicinity of the active site which is clearly understood from Fig. 3a.

Based on the fact that azoles exhibit antifungal activity by inhibition of a cytochrome P450 enzyme, lanosterol-14-α-demethylase during the biosynthesis of ergosterol leading to accumulation of 14-α-sterols in the fungal cell membrane causing destabilization and membrane degradation, it was thought worthwhile to investigate the binding mode of the highest active test compound, 6c, 2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide in the active site of chimeric enzyme of cytochrome P450-14-α-sterol demethylase from *Candida albicans* (*Candida* P450DM) (chimeric 1EA1).

Binding mode analysis of test compound 6c in the active site of chimeric 1EA1 (Fig. 4a) showed that the test compound was very close to the porphyrin ring, HEM 470, similar as that of the cocrystallized ligand Fluc. Docking poses of the test compound suggest the presence of active site residues leu321, tyr76 as in case of Fluc (Fig. 4b).

CONCLUSION

A series of twelve 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives (6a-6l) were synthesized and evaluated for their *in vitro* antimicrobial potency and found to be potent to moderately active in inhibiting the pathogenic growth. The significant antimicrobial property may be attributed to the putative substructure of triazole (which itself can form ionic interaction with the various microbial target; the most acceptable is lanosterol-14α-demethylase in *Candida albicans*) and the salient feature of the hydroxamic group. The aryl group with its substituent leaves a mixed opinion with respect to the biological interaction. The electron donating ability of methyl, mercapto, amino group strengthen the binding affinity of the aryl group with the active site of the target might be one of the strong supporting evidence for their potency, but in the same ground how this nitro group being a strong electron group helps in lifting the chemotherapeutic index is difficult to establish. Therefore, the series requires to be well extended. Thus, it could be concluded that the synthesized 1,2,4-triazole-linked hydroxamic acid derivatives with electron donating groups at 5-position of the 1,2,4-triazole moiety may provide a therapeutically effective chemical framework from which potential antimicrobial agents may be developed further. Therefore, further optimizations of this prototypical molecular framework with some diversified molecular fragments may generate new drug entities having potent antimicrobial activity.

ACKNOWLEDGMENTS

This experimental work was supported by the Indian Council of Medical Research (ICMR), India. Mousumi Das is a senior research fellow of ICMR (No. 45/9/13-PHA/BMS). The authors are grateful to UPE Phase - II, University Grants Commission, Government of India, for providing instrumental support.

AUTHORS' CONTRIBUTIONS

All authors have made considerable contributions to the work reported in the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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Antioxidant and anticancer activity of synthesized 4-amino-5-((aryl substituted)-4H-1,2,4-triazole-3-yl) thio-linked hydroxamic acid derivatives

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Keywords
1,2,4-triazole; anticancer; antioxidant; hydroxamic acid derivatives; *in vivo* study

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Received March 23, 2019
Accepted May 21, 2019

doi: 10.1111/jphp.13131

Abstract

Objectives The antioxidant and anticancer activity of twelve 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives were evaluated.

Methods Previously synthesized 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide and 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (**6a–6l**) were evaluated for in vitro antioxidant and in vivo anticancer activity. MDA-MB-231, MCF-7 and HCT 116 cell lines were used to evaluate IC_{50} values, in vitro. Ehrlich ascites carcinoma (EAC)-induced mice model was used to evaluate in vivo anticancer potential. Different biological markers were examined for drug-related toxicities.

Key findings Compound **6b** revealed more potent antioxidant property among all tested compounds, even than the ascorbic acid. The IC_{50} values of compound **6b** were found to be 5.71 ± 2.29 μg/ml (DPPH assay) and 4.12 ± 0.5 μg/ml (ABTS assay). Histopathology of liver sections of drug-treated mice was evaluated. Survival analysis showed that compound **6b** could increase the life span as of the standard drug.

Conclusions After the assessment of all in vivo anticancer study related data, it was found that compound **6b** possess superior anticancer potency in terms of efficacy and toxicity. From this experimental design, it could be concluded that further modification of this prototypical structure will lead to develop more potent antioxidant as well as an anticancer agent in the future.

Introduction

Heterocyclic compounds have been used to be the most researched chemical entity in the area of organic chemistry, having wide verities of application in the field of pharmaceuticals to agro-industries.[1] Among the heterocyclic compounds, nitrogen-containing heterocycles like triazole grab the attention of the scientific community over the decades. 1,2,4-Triazoles is an interesting isomer of triazoles having a diversified pharmacological activities such as anticancer, antidepressant[2], antibacterial[3], antifungal[4] anti-inflammatory, antileishmanial[5] and antiviral activities[6]. It could be found from different experimental design associated with the 1,2,4- triazole that it also possesses superior antioxidant activity. Cetin and Geçibesler[7] demonstrated that phenol and pyridine substituted 1,2,4-triazole attributed exceptional antioxidant property. Pokuri *et al.*[8] have designed and synthesized different derivatives of 1,2,4 triazoles and found significant antioxidant activity over the standard ascorbic acid. Karrouchi *et al.*[9] have synthesized a series of pyrazole containing derivatives of 1,2,4-triazoles and found significant antioxidant activity for all of the synthesized compounds.

Hydroxamic acids (R-CO-NH-OH) are also an important class of chemical compounds which could be derived from hydroxylamine (NH_2OH) and carboxylic acids (R-COOH). This class of compounds has the ability to chelate metal ions and known to have multiple biological activities.[10] They found to have potent antioxidant activities. Like, Koncic *et al.* have synthesized hydroxamic acid derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) and found significant radical scavenging, antioxidant and metal chelating activities. They have found that N-methylhydroxamic acid of diclofenac showed potent

radical scavenging activities.[11] Bernardo *et al*.[10] have synthesized a series of hydroxamic acid derivatives with different chemical groups like aliphatic, aromatic and amino acids and found high antioxidant activity of almost all of them.

Besides the antioxidant potential of hydroxamic acid derivatives, antitumour or anticancer activity is also well established. Suberoylanilide hydroxamic acid (SAHA) is a well-known example of hydroxamic acid derivatives. SAHA is a potent histone deacetylase inhibitor and clinically approved to treat cutaneous T-cell lymphoma.[12] However, the anticancer efficacy and apoptosis effect of SAHA were evaluated against different types of cancer.[13,14] Zhao *et al*. reported the SAHA as potent antitumour agent for large-cell lung carcinoma. It has been found from the work that SAHA could inhibit the cell proliferation by arresting the cell cycle G2/M phase in a dose- and time-dependent manner. They also found potent tumour suppression in nude mouse xenograft model.[12] According to Peela *et al*., against the treatment of solid tumours SAHA have provided conflicting results even in clinical trials. However, they have found significant inhibition of cellular migration as well as decreased microtubule polarization in case of SAHA against breast cancer cell line, SUM159.[15] Besides SAHA, there were several derivatives of hydroxamic acids have shown effective anticancer efficacy. Like, Tardibono and Miller[16] have synthesized several hydroxamic acid containing 1,4-Benzodiazepines and found to have good anticancer activity. A series of diaryl ether hydroxamic acids were synthesized by Zhu *et al*.,[17] and some of them have shown inhibitory activity against four cancer cell lines.

The number of research work to develop superior antioxidant as well as anticancer agents implies the necessity to treat free radical associated diseases to life-threatening cancer. Considering the above fact and in a search for new antioxidant and anticancer agent, we have evaluated the antioxidant and anticancer activity of a series of 4-amino-5-((aryl substituted)-4H-1,2,4-triazole-3-yl)thio-linked hydroxamic acid derivatives, which have been synthesized and reported by us recently.[18] In this experimental design, we have evaluated the antioxidant potency by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) radical cation scavenging activities and total antioxidant capacity. Among the different cancer types, breast and colon cancer are the global problem now a days. Search for a new anticancer lead, in vitro anticancer activity was evaluated against different breast cancer cell line like MDA-MB-231, MCF-7 as well as against HCT 116, which is a very common cell line for colorectal carcinoma. In vivo antitumour efficacy was evaluated against Ehrlich ascites carcinoma (EAC)-induced mice model.

Materials and Methods

Materials

See the Appendix S1.

Methods

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl radical scavenging activities. Antioxidant activity of the synthesized compounds (**6a–6l**) was evaluated by determining its scavenging ability against DPPH radical.[19] 100 µl of DPPH (0.1 mM) in ethanol was added in a micro-titer plate. 50 µl of different concentrations (2.5–400 µg/ml) of the tested compounds was added further. Milli-Q water was used to prepare the control. Then, the plate was incubated in dark for 30 min at room temperature. The absorbance was measured at 517 nm using a micro-plate reader (SpectraMax M5; Molecular Devices, San Jose, CA, USA). To evaluate the contribution of the sample colour itself, samples without DPPH were prepared and absorbance was measured similarly. Ascorbic acid was taken as standard. The free radical scavenging activity was determined by the following equation,

$$\%\,\text{Inhibition} = \frac{A_c - A_s}{A_c} \times 100\%$$

where A_c denoted the absorbance of control and A_s denoted the absorbance of sample. IC_{50} value of the synthesized compounds was determined. IC_{50} value is the half maximal inhibitory concentration to measure the potency of a substance in inhibiting 50% absorbance of initial DPPH concentration.

Total antioxidant capacity

The total antioxidant capacity (TAC) of the synthesized compounds (**6a–6l**) was evaluated by phosphomolybdenum method.[20] This method relies on the fact that if a sample having antioxidant property then it could reduce Molybdenum(VI) to form Molybdenum(V). In acidic pH, the green phosphate/Molybdenum(V) complex was formed and the intensity of which could be measured at 695 nm. Briefly, 20 µl of the tested compounds of varying concentrations (2.5–320 µg/ml) was mixed with 200 µl of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated for 90 min at 95 °C and cooled down to room temperature. The absorbance was measured at 695 nm against a blank. The antioxidant activity was calculated from the

standard curve of the gallic acid and expressed as mg gallic acid equivalent (GAE)/g.

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activities. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^+$) scavenging activity was assessed.[21] ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) to produce $ABTS^+$ solution and kept in the dark for 16 h at room temperature. The solution was then diluted with phosphate buffer saline (PBS; pH 7.4) to get an absorbance of ~0.750 at 734 nm. 180 µl of diluted $ABTS^+$ was mixed with 20 µl of the synthesized compounds at different concentrations (1.56–50 µg/ml) and allowed to react at 30 °C for 20 min. Sample blank was prepared by $ABTS^+$ solution and ethanol. The percentage inhibition of absorbance was measured at 734 nm. Ascorbic acid was used as a reference standard. The radical scavenging activity of the samples was calculated by the similar equation used for DPPH assay.

Anticancer activity

In vitro anticancer activity. MTT assay was employed to carry out in vitro anticancer activity. All the synthesized compounds were tested against HCT 116, MDA-MB-231 and MCF-7 cell lines. Cells were cultured in 10% FCS supplemented DMEM medium and maintained in a 5% CO_2 incubator at 37 °C. Briefly, 100 µl of supplemented media containing 5×10^3 cells were seeded into the predetermined wells of 96-well plates and kept into 5% CO_2 incubator for 24 h at 37 °C. After that media was replaced carefully with 100 µl of fresh media containing different predetermined concentrations (2.5–320 µg/ ml for MCF-7 and HCT 116, and 5–100 µg/ml for MDA-MB-231) of tested compounds and kept for another 24 h at previous incubation condition followed by careful removal of media, and 10 µl of MTT reagent (5 mg/ml) was added and incubated in dark for 4 h. 100 µl of DMSO was added to dissolve the formazan crystals after removing the supernatant from each well and followed by the mechanical shaking for 10–15 min. Absorbance was taken at 570 nm. Percentage inhibition was calculated by the following equation,

$$\%\,\text{Inhibition} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100\%$$

where A_{control} and A_{test} were the absorbance of control and test, respectively.

Study of in vivo anticancer activity

Experimental animals. Female swiss albino mice (20–25 g body weight) were used to carry out the in vivo experiments. The animals were kept at standard laboratory conditions (Temp. 25 ± 2 °C and humidity 55 ± 10%) under alternating light and dark environment (12/12 h). Animals were fed with standard food pellet diet, and drinking water was provided ad libitum. The guidelines of Institutional Animal Ethics committee, Jadavpur University, India (Ref. No. AEC/PHARM/1401/2014; dated 17.11.2014) were followed to carry out all experimental procedures. The animals were kept for one week in laboratory condition before initiation of these in vivo experiments.

In vivo tumour cell transplantation. The Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The anticancer effect of the tested compounds (**6b**, **6j**, **6l**) was evaluated in vivo against EAC cell line as the EAC cells induced mammary gland carcinoma in female mice mimic the breast cancer model in human.[22] The ascitic fluids were collected from peritoneal cavity by sterile syringe and kept in a sterile petri plates followed by repeated washing with PBS to remove the non-adherent cells. Then, the ascitic fluids were centrifuged, and the pellets were suspended in sterile isotonic saline. Cell viability was checked by trypan blue exclusion assay, and viability was assessed to be 95%, and these viable cells were used to carry out the further experiments.[23,24]

Experimental design

The animals were divided into twelve groups containing six animals ($n = 6$) in each group. All the experimental animals of each group were injected with EAC cells (2×10^6 cells/ mouse) through intraperitoneal route, except 'Group I', and the day of cell inoculation was treated as 'day 0' on which no treatment was given. The group distributions were as follows:

Group I: was denoted as normal control (normal saline, 5 ml/kg body weight, i.p). Animals were injected with sterile distilled water for injection.

Group-II: was denoted by EAC control. Animals were treated with EAC cells by intraperitoneal injections from day 1 to day 10.

Group III: was designed as reference group. Animals were treated with standard drug [5-Fluoro Uracil (5-FU) 20 mg/ kg body weight] by intraperitoneal injections from day 1 to day 10.

Group IV: Animals were treated with tested compound, **6b** (25 mg/kg body weight) from day 1 to day 10.

Group V: Animals were treated with tested compound, **6j** (25 mg/kg body weight) from day 1 to day 10.

Group VI: Animals were treated with tested compound, **6l** (25 mg/kg body weight) from day 1 to day 10.

Group VII: Animals were treated with tested compound, **6b** (50 mg/kg body weight) from day 1 to day 10.

Group VIII: Animals were treated with tested compound, **6j** (50 mg/kg body weight) from day 1 to day 10.

Group IX: Animals were treated with tested compound, **6l** (50 mg/kg body weight) from day 1 to day 10.

Group X: Animals were treated with tested compound, **6b** (125 mg/kg body weight) from day 1 to day 10.

Group XI: Animals were treated with tested compound, **6j** (125 mg/kg body weight) day 1 to day 10.

Group XII: Animals were treated with tested compound, **6l** (125 mg/kg body weight) day 1 to day 10.

Survivability study

Life span of the EAC-induced mice was significantly lower compared with the healthy one. To study the effect of compounds **6b**, **6j** and **6l** on lifespan of EAC-induced mice, we performed survivability assay after treatment with three compounds (**6b**, **6j** and **6l**) at their higher dose use for the in vivo study (125 mg/kg) and another group was treated with 5-FU (20 mg/kg). To carry out the experiment, six mice were taken in each group. The survivability was determined by following equation

$$\%\,\text{Survivability} = \frac{M_{\text{treated}} - M_{\text{control}}}{M_{\text{control}}} \times 100\%$$

where M_{treated} and M_{control} were the number of days' mice survived after treatment and survived in case of control, respectively. Kaplan–Meir survival analysis was carried out using Graph Pad Prism (GraphPad Software, La Jolla, CA, USA).

Histopathological study

On 11th day, liver tissues were dissected out from each group of sacrificed animals and preserved in formalin solution immediately. The harvested tissues were embedded in paraffin wax followed by cut into thin sections by using microtome instrument. The sections were then placed on clean glass slides to counter stain with haematoxylin and eosin (H&E) where haematoxylin stains the nucleus with blue colour, whereas cytoplasm stained with eosin which was red in colour. After staining, the glass slides containing liver sections of different groups were subjected to take images under light microscope.

Statistical analysis

Experimental data were presented as mean ± standard deviation. One-way analysis of variance was used to determine the statistical significance. $P < 0.01$ was considered as statistically significant, whereas $P > 0.05$ was considered to be statistically not significant.

Results and Discussion

Synthesized compounds

Twelve 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives were synthesized and reported by our group recently.[18] Detailed synthetic procedure was discussed there along with their spectral characterization, that is FTIR, H^1 NMR and mass spectral analysis were carried out. Figure 1 displayed the chemical structures of the previously synthesized compounds of which the in vitro antioxidant and in vivo anticancer studies were evaluated in this experimental work.

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activities

2,2-Diphenyl-1-picrylhydrazyl assay is one of the most rapid and simple technique to determine the antioxidant efficacy of tested compounds.[25] $DPPH^{\cdot}$ is a stable free radical which is commercially available. The theory behind the assay is that $DPPH^{\cdot}$ accept hydrogen atom to pair with free odd electron from any tested compound having hydrogen donating capability, and thus, the tested compound is said to be an antioxidant. In this study, compound **6a–6l** was evaluated for their free radical scavenging activity in the presence of DPPH where ascorbic acid was used as standard antioxidant. The absorption of purple colour DPPH could be measured by UV spectrophotometer at a wavelength of 517 nm. However, in presence of any antioxidant, the colour turns into yellow. The reduction in colour intensity reflects the ability of an antioxidant as a radical scavenger.[26]

The IC_{50} values were measured for tested compounds and represented in Table 1. Here, the IC_{50} value denoted the effective concentration at which 50% of the initial DPPH was scavenged. The radical scavenging activity of the tested compounds was compared with the positive control, ascorbic acid. It was found from Table 1 that compound **6a–d**, **6k** and **6l** have potent antioxidant activity with IC_{50} values of 10.79 ± 3.76, 5.71 ± 2.29, 8.46 ± 2.96, 7.52 ± 1.55, 8.07 ± 2.34 and 5.02 ± 1.68 μg/ml, respectively, compared with the standard ascorbic acid having the IC_{50} of 15.15 ± 1.72 μg/ml. The superior antioxidant activity demonstrated that pyridyl, *p*-amino benzyl and *m*-nitro benzyl substitutions at R^1 position were important. As they provide a

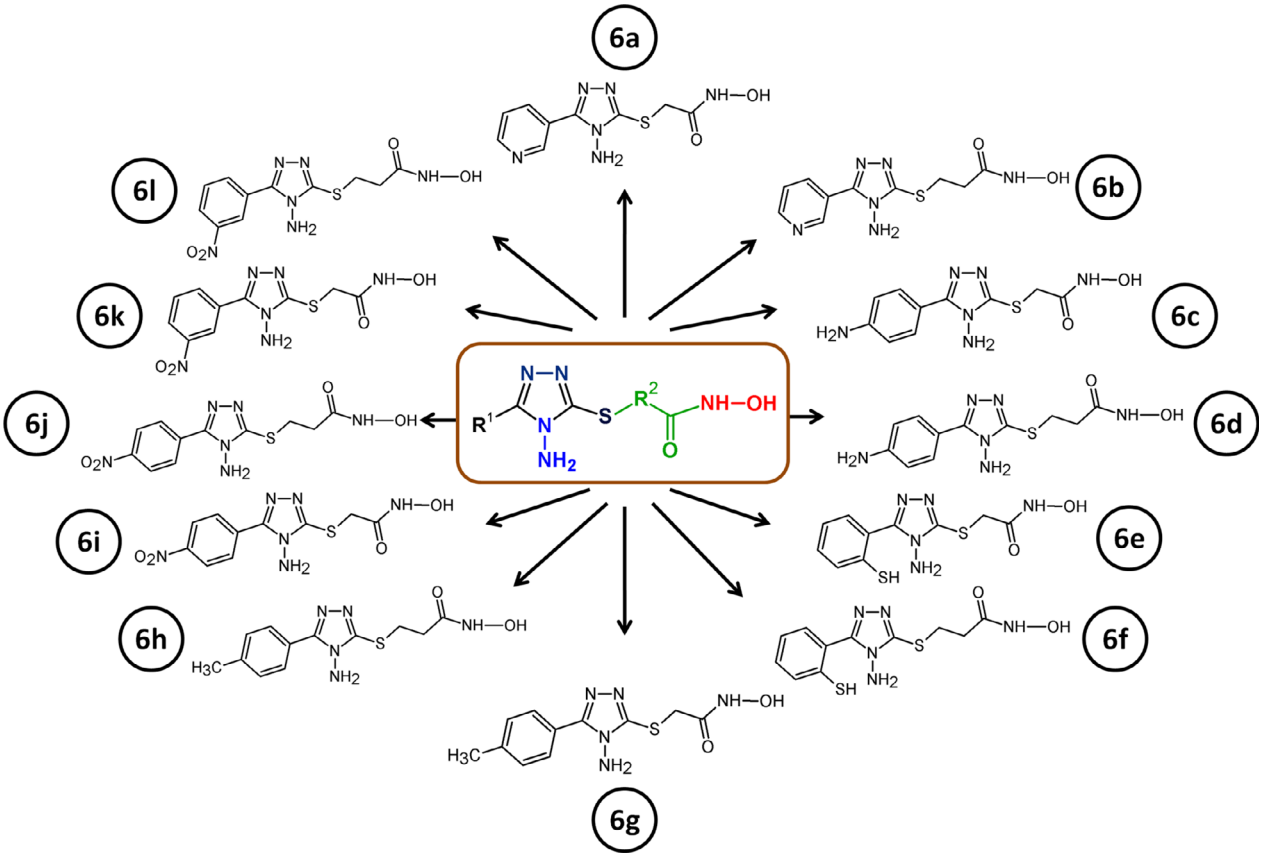

Figure 1 Twelve 5-substituted-4-amino-1, 2, 4-triazole-linked hydroxamic acid derivatives. [Colour figure can be viewed at wileyonlinelibrary.com]

resonance effect in the structure of the tested compounds, making them more reactive which could be justified by the work done by Cetin and Geçibesler,[7] who reported that pyridine substituted 1,2,4-triazole compounds have a better prospect to be an antioxidant.

ABTS [2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical cation scavenging activities

It was another method to determine the antioxidant activity of tested compounds. Except compound **6e** and **6f**, all other have shown superior to moderate antioxidant activities compared with the standard, ascorbic acid (55.84 ± 0.23 μg/ml). Compounds **6b** showed excellent ABTS radical cation scavenging activity with IC_{50} value of 4.12 ± 0.5 μg/ml, and compounds **6a**, **6c**, **6d**, **6k** and **6l** were also active as they nearly fully scavenged ($ABTS^{+}$) with IC_{50} value of 14.65 ± 0.55, 23.53 ± 3.62, 16.41 ± 1.02, 21.82 ± 0.69 and 18.54 ± 3.92 μg/ml, respectively. It was observed that the reaction was very fast and completed within 1 min. During the reaction time, the changes in absorbance were very negligible. It was also found that like DPPH assay results, ABTS assay has also shown that compound **6a–d**, **6k** and **6l** have superior radical scavenging activities which could be due to the similar effect described in case of DPPH assay.

Table 1 DPPH and ABTS radical scavenging activities of the tested compounds

Tested compounds	IC_{50} (μg/ml)	
	DPPH	ABTS
6a	10.79 ± 3.76^{ns}	14.65 ± 0.55**
6b	5.71 ± 2.29**	4.12 ± 0.50**
6c	8.46 ± 2.96*	23.53 ± 3.62**
6d	7.52 ± 1.55*	16.41 ± 1.02**
6e	99.64 ± 0.82**	84.46 ± 1.96**
6f	118.22 ± 0.83**	71.38 ± 3.07**
6g	75.58 ± 3.21**	49.40 ± 0.68*
6h	68.29 ± 4.21**	45.30 ± 2.65**
6i	69.96 ± 3.36**	41.57 ± 2.91**
6j	47.85 ± 3.49**	33.74 ± 2.02**
6k	8.07 ± 2.34*	21.82 ± 0.69**
6l	5.02 ± 1.68**	18.54 ± 3.92**
Ascorbic acid	15.15 ± 1.72	55.84 ± 0.23

Data expressed as mean ± SD, $n = 3$; significant difference was calculated against control; $^{**}P < 0.01$, $^{*}P < 0.05$, ^{ns}not significant.

Total antioxidant capacity

A green phosphate/Molybdenum (V) complex was produced in the phosphomolybdenum method, and the absorption was measured at 695 nm. The absorbance value was proportional to antioxidant potency of the tested compounds. The reducing capacity or electron donating potency of the antioxidants to molybdenum was evaluated in the assay. The reducing ability of a chemical entity may play as a remarkable indicator of its potential antioxidant activity. The total antioxidant activity was expressed as mg GAE/g of the tested compounds. The compounds **6a** (637.5 ± 4.42), **6b** (233.59 ± 1.10), **6d** (124.21 ± 5.52), **6e** (348.44 ± 4.42), **6f** (172.66 ± 1.10) and **6k** (148.44 ± 4.42) have shown greater total antioxidant capacity compared with the other tested compounds **6c** (61.72 ± 3.31), **6g** (13.28 ± 1.11), **6h** (20.31 ± 4.41), **6i** (62.5 ± 2.21), **6j** (76.56 ± 4.42) and **6l** (61.72 ± 3.31). Among all of them, compounds **6a**, **6b** and **6e** showed highest activity.

Anticancer activity

In vitro assay

Among the other types of cancer, breast cancer is common among women. According to world health organization (WHO), each year about 2.1 million women affected by it globally. Recent reports of 2018 suggested that about 0.6 million women died from breast cancer which is nearly 15% of all cancer-related deaths among women.[27] Besides the breast cancer, according to the National Cancer Institute of United States, 0.15 million new cases of colon cancer could be identified in the year of 2019 which is about 8.3% of all new cancer cases. Among them, almost 50,000 estimated deaths were happened which is 8.4% of all cancer-related death. The statistical data itself indicated the necessity to develop new anticancer agents. By accepting this challenge, scientist from all over the globe have been tried to develop new leads over decades and have found 1,2,4-triazole and hydroxamic acid derivatives have a huge potency to be an antibreast cancer agent. We have recently synthesized and identified twelve derivatives of 4-amino-5-((aryl substituted)-4H-1, 2, 4-triazole-3-yl (Figure 1). The synthesized compounds were explored for their in vitro anticancer activity using MTT assay against three human cancer cell lines, among them two were breast cancer cell lines, like MCF-7 and MDA-MB 231, whereas another one was colon cancer cell line, that is HCT 116. The percent inhibition of the three types of cancer cells, that is MCF-7 (Figure 2a), MDA-MB-231 (Figure 2b) and HCT116 (Figure 2c) by the tested compounds at their different concentrations, could be observed at Figure 2. It was found that majority of the tested compounds exhibit anticancer

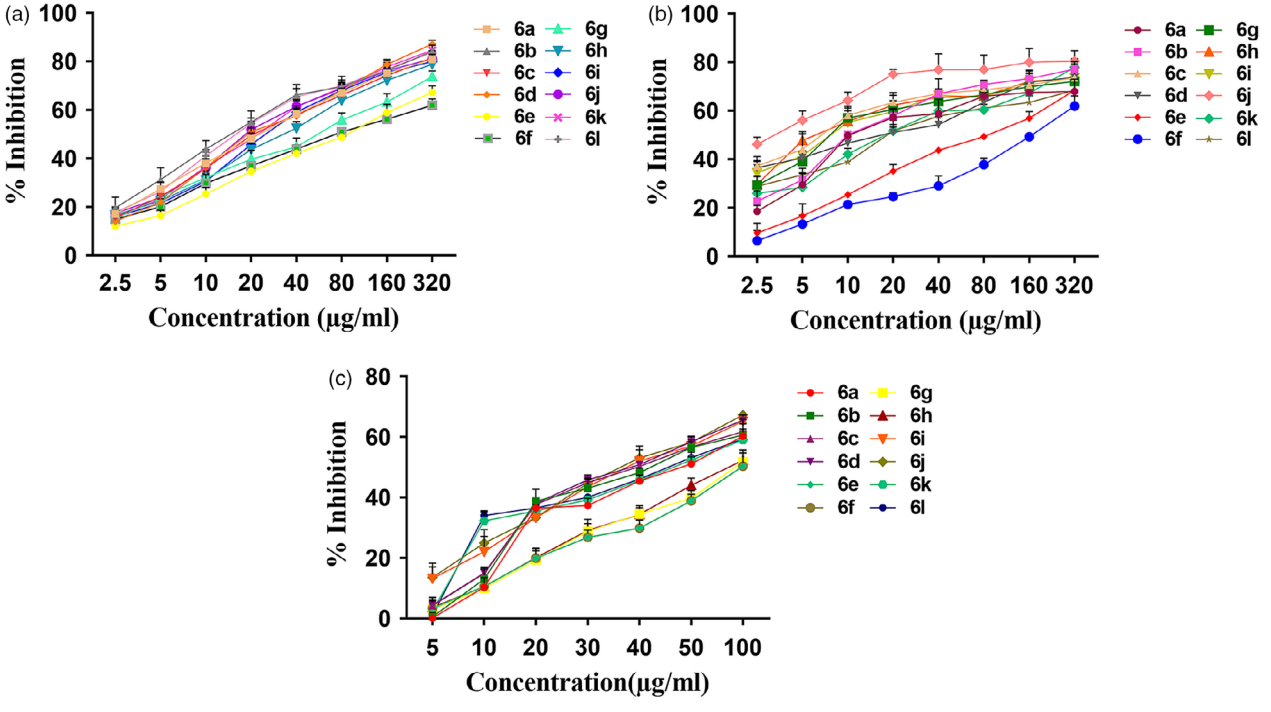

Figure 2 Percentage inhibition of compounds **6(a–l)** against (a) MCF-7 cell line, (b) MDA-MB-231 cell line and (c) HCT 116 cell line. [Colour figure can be viewed at wileyonlinelibrary.com]

activity with IC_{50} values ranging from 3.74 ± 0.79 to 168.73 ± 7.79 μg/ml (Table 2). Some of them exhibited promising anticancer activity against the three cancer cell lines.

To compare the effect of the tested compounds on highly invasive (MDA-MB-231), poorly invasive (MCF-7) breast cancer cells and human colon carcinoma cells (HCT 116), we treated these cells with the synthesized compounds (0–320 μg/ml) for 24 h and percent inhibitions were determined. From which IC_{50} values were calculated and tabulated as Table 2.

Table 2 IC_{50} values of synthesized compounds against MCF-7, MDA-MB-231 and HCT 116 cell line

Tested compounds	IC_{50} (μg/ml)		
	MCF-7	MDA-MB-231	HCT 116
6a	23.77 ± 4.28	47.15 ± 1.48	10.82 ± 1.68
6b	15.85 ± 3.52	41.82 ± 1.20	10.408 ± 0.75
6c	22.27 ± 4.68	39.77 ± 1.11	14.69 ± 4. 37
6d	20.13 ± 2.88	38.49 ± 0.46	6.58 ± 1.58
6e	90.61 ± 9.56	98.09 ± 4.22	168.73 ± 7.79
6f	74.89 ± 9.6	92.19 ± 9.47	85.14 ± 4.76
6g	58.23 ± 6.29	92.00 ± 4.43	8.13 ± 0.39
6h	34.21 ± 5.99	86.27 ± 8.28	6.52 ± 1.33
6i	26.27 ± 4.15	38.31 ± 4.27	8.52 ± 1.57
6j	19.02 ± 2.08	37.02 ± 4.35	3.74 ± 0.79
6k	23.07 ± 3.63	44.59 ± 2.48	19.24 ± 2.22
6l	16.69 ± 1.60	43.80 ± 2.16	19.18 ± 3.72

Data expressed as mean ± SD, $n = 3$.

From Table 2, it could be found that all of the tested compounds have shown better activity (according to their IC_{50} values) against MCF-7 breast cancer cell lines rather than the triple negative breast cancer cell line, MDA-MB-231. Among them, compounds **6b**, **6j** and **6l** have shown better inhibition against MCF-7 with the IC_{50} values of 15.85 ± 3.52, 19.02 ± 2.08 and 16.69 ± 1.60 μg/ml, respectively. Besides the activity against two different breast cancer cell lines, the tested compounds have shown superior inhibition against HCT 116 with a minimum IC_{50} of 3.74 ± 0.79 μg/ml for compound **6j**. It could be very interesting to found that increasing the linear chain length at R^2 position made the compounds more active, that is compounds **6b**, **6d**, **6f**, **6h**, **6j** and **6l** were more active than compounds with methyl substitution at R^2 position like compound **6a**, **6c**, **6e**, **6g**, **6i** and **6k**, which in turn indicated that the linker group (R^2) of compounds **6b**, **6d**, **6f**, **6h**, **6j** and **6l** was probably beneficial to its activity. The tested compounds were designed and synthesized with two points of diversity, that is, variation in substituent on the surface recognition moiety (phenyl ring) and variation in the linker group. However, the better anticancer activity of compounds **6b**, **6j** and **6l** among the twelve tested compounds against MCF-7 reflects the importance of the substituent like pyridyl, *p*-nitrobenzene and *m*-nitrobenzene on the surface recognition moiety played important role in their biological activity. The results could also be justified by the research done by Saha *et al.* where they have synthesized hydroxamic acid linked 5-aryl substituted 4-amino-

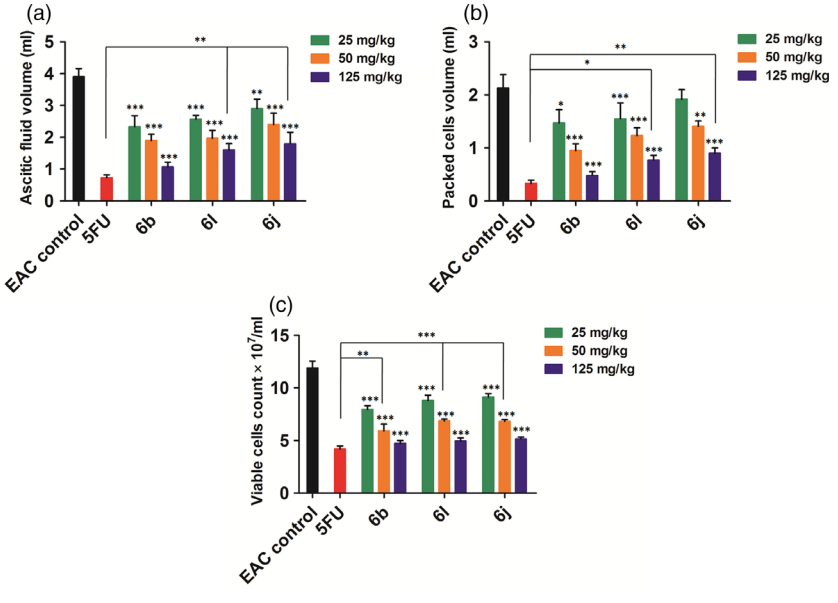

Figure 3 (a) Ascitic fluid volume; (b) packed cell volume; and (c) viable cell count was carried out for compounds **6b**, **6j** and **6l** at different concentrations like 25, 50 and 125 mg/kg body weight. 5-Fluoro Uracil was used as standard. Data expressed as mean ± SD, $n = 3$; significant difference was calculated against control; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

1,2,4-triazole analogues and found to have similar binding interaction with receptor, HDAC8, like standard SAHA.[28] Hassan *et al*. also demonstrated that aryl substitution at the 5-position of 1,2,4,-triazole ring could develop potent anticancer agent.[29] Considering the in vitro anticancer data generated for tested compounds against the breast cancer cell line like MCF-7, compounds **6b**, **6j** and **6l** were further selected to evaluate their in vivo anticancer activity against EAC-induced mice model.

In vivo experiment

Ascitic fluid, packed cells and viable cell count. Ehrlich ascites carcinoma is a very rapid growing and produces liquid tumour in abdominal region. This type of carcinoma is very aggressive in nature results elevated vascular permeability, higher cellular migration and formation as well as accumulation of ascitic fluid. As the ascitic fluid act as a direct source of nutrition for the tumour cells, it plays a very important role in tumour formation. So, minimizing the volume of ascitic fluid, packed cells as well as viable cell count indicated the efficacy of a compound as an anticancer agent. In this experiment, compounds **6b**, **6j** and **6l** showed significant dose-dependent reduction in ascitic fluid volume, packed cell volume, viable cell count compared with EAC control animals (Figure 3). When the volume of ascitic fluid upon the treatment of compounds **6b**, **6l** and **6j** compared with the standard, it could be found that compound **6b** at a concentration of 125 mg/kg have shown no significant differences, indicated the potency to decrease the volume of ascitic fluid compared with that of the standard (Figure 3a). Similar results have been found in case of packed cell volume (Figure 3b) and viable cell count (Figure 3c), and compound **6b** (125 mg/kg) showed comparable potency with standard.

Haematological parameters. When haematological parameters were measured, notable reduction in haemoglobin level (10.82 ± 0.42 g/dl), RBC counts (5.41 ± 0.47) and elevated WBC counts (11.81 ± 0.51) were observed in case of EAC control group of animals (Table 3). The treated group containing compounds **6b**, **6j** and **6l** showed dose-dependent increase in haemoglobin (14.44 ± 0.43, 12.90 ± 0.42 and 14.17 ± 0.51 g/dl), RBC (8.17 ± 0.24, 6.92 ± 0.19 and 8.10 ± 0.38) counts and decreased in WBC counts (6.70 ± 0.20, 7.82 ± 0.24 and 6.30 ± 0.32) nearer to

Table 3 Effect of compounds **6b**, **6j** and **6l** on different blood parameters of Ehrlich ascites carcinoma cell-bearing mice

	Haemoglobin (g/dl)	RBC (/ml)	WBC (/ml)
Control	15.82 ± 0.62	8.78 ± 0.44	6.79 ± 0.41
EAC control	10.82 ± 0.42	5.41 ± 0.47	11.81 ± 0.51
6b (25 mg/kg)	11.137 ± 0.35***	6.02 ± 0.34**	9.22 ± 0.24***
6b (50 mg/kg)	12.03 ± 0.45***	6.71 ± 0.14**	8.65 ± 0.22***
6b (125 mg/kg)	14.44 ± 0.43*	8.17 ± 0.24	6.70 ± 0.20
6l (25 mg/kg)	10.97 ± 0.38***	5.51 ± 0.32**	10.14 ± 0.51***
6l (50 mg/kg)	11.36 ± 0.54***	6.65 ± 0.61**	8.85 ± 0.27***
6l (125 mg/kg)	14.17 ± 0.51*	8.10 ± 0.38	6.30 ± 0.32
6j (25 mg/kg)	10.95 ± 0.13***	5.55 ± 0.12**	9.98 ± 0.27***
6j (50 mg/kg)	11.59 ± 0.39***	6.14 ± 0.68**	8.93 ± 0.37***
6j (125 mg/kg)	12.90 ± 0.42***	6.92 ± 0.19***	7.82 ± 0.24*

Data expressed as mean ± SD, $n = 3$; significant difference was calculated against control; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Table 4 Effect of treatment with compound **6b**, **6j** and **6l** on different biochemical parameters of Ehrlich ascites carcinoma cell-bearing mice

	SGPT (U/L)	SGOT (U/L)	Albumin (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Bilirubin (mg/dl)
Control	44.78 ± 4.67	147.78 ± 8.54	3.05 ± 0.35	0.39 ± 0.02	40.12 ± 2.83	0.10 ± 0.01
EAC control	89.68 ± 8.33	294.77 ± 11.32	1.55 ± 0.21	0.83 ± 0.07	63.47 ± 6.93	0.36 ± 0.05
6b (25 mg/kg)	64.63 ± 5.93**	187.57 ± 10.20**	2.08 ± 0.15**	0.52 ± 0.03**	50.03 ± 3.19*	1.98 ± 0.28***
6b (50 mg/kg)	58.78 ± 5.48*	174.95 ± 14.14	2.55 ± 0.16	0.45 ± 0.05	47.77 ± 5.92	1.78 ± 0.25***
6b (125 mg/kg)	55.43 ± 5.07	170.45 ± 15.09	2.78 ± 0.31	0.42 ± 0.04	44.52 ± 4.03	1.62 ± 0.29
6l (25 mg/kg)	74.58 ± 5.39***	199.17 ± 9.00***	2.20 ± 0.20**	0.59 ± 0.05***	55.28 ± 5.18**	2.68 ± 0.34***
6l (50 mg/kg)	65.78 ± 4.69**	190.32 ± 11.44**	2.30 ± 0.58	0.50 ± 0.06	50.12 ± 2.76	2.32 ± 0.34***
6l (125 mg/kg)	60.38 ± 4.23*	174.50 ± 14.48	2.45 ± 0.23	0.45 ± 0.05	46.75 ± 5.23	2.15 ± 0.41
6j (25 mg/kg)	77.03 ± 5.44***	212.18 ± 8.82***	1.90 ± 0.15**	0.63 ± 0.03***	56.90 ± 3.28**	2.83 ± 0.29***
6j (50 mg/kg)	73.33 ± 4.52***	197.83 ± 9.54**	2.05 ± 0.15*	0.56 ± 0.04**	50.67 ± 3.38*	2.52 ± 0.30***
6j (125 mg/kg)	67.63 ± 6.03**	178.78 ± 14.78	2.37 ± 0.33	0.47 ± 0.05	48.07 ± 4.94	2.27 ± 0.42

Data expressed as mean ± SD, $n = 6$; significant difference was calculated against control; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

normal value. Like the result obtained for ascitic fluid volume, packed cell volume and viable cell count of compound **6b**, here also a similar trend could be observed at the dose of 125 mg/kg. From the statistical analysis comparing the results of control, it was clearly observed that compound **6b** and **6l** have shown almost negligible significant difference which indicated the fact that pyridyl and m-nitrobenzene moiety at R^1 position was important to exert anticancer potency of the tested compounds.

Biochemical parameters. SGPT, SGOT, creatinine, bilirubin and urea level were significantly increased in the serum after development of ascetic carcinoma in compared with the normal mice, whereas albumin level was significantly reduced. Liver and kidney toxicities levels almost back to normal after treatment with compounds **6b** and **6j** (dose of 125 mg/kg) in mice having ascetic carcinoma in compared to the normal mice (Table 4). It could be observed from Table 4 that SGPT, SGOT, creatinine, urea and bilirubin level of compound **6b** markedly decreased than compound **6j** at the dose of 125 mg/kg body weight. Although there is no significant different in the result obtained from compound **6b** and **6l** (125 mg/kg), the change of biochemical markers compared with the control is more significant than **6l–6b** at even higher dose.

Survivability study

It could be found from Figure 4a that survivability has seriously been affected by the EAC induction. To evaluate the effect of the dose, used to treat the animals on their reduced survivability, was the main objective of this experiment and found very interesting results. Compared with the effect of 5-FU treatment on the percent survival, the tested compounds have shown promising activity, especially compound **6b**. From Figure 4b, it could be clearly observed that the rate of survivability has been significantly increased in case of compound **6b** compared with the other tested compounds, even from the standard. It was found that 5-FU elevated the life span of the EAC bearing mice to almost 191%, whereas compound **6b** at its higher experimental dose of 125 mg/kg body weight, elevated it to 205%. Though the life span elevation due to the treatment of compound **6b** was statistically non-significant compared with the standard, still it could be said that it produces similar effectiveness among the other two tested compounds **6l** and specially **6j**. The per cent survivability of compound **6j** was markedly declined compared with the compound **6b** which could be justified by the results obtained in Tables 3 and 4. From Table 3, it could be found that the haemoglobin, RBC and WBC count were not significantly increased compared with the compound **6b** and the scenario was same in case of biological markers presented in Table 4.

Histology

Figure 5 showed the effect of treatment on the liver, as liver is the most effected organ in EAC bearing mice. In this study, we not only have shown the effect of different compounds but also have shown the effect of different doses on the particular organ. Figure 5a represents the liver section of normal control. Normal hepatocytes with vesicular nuclei could be observed with normal anatomy of sinusoid capillaries. Hepatocytes were evenly separated by sinusoids, whereas liver section of EAC bearing mice (Figure 5b) showed vacuolated hepatocytes along with steatosis. Dilated sinusoids have also been found in EAC control. Effect of different doses of compound **6b** could be observed in Figure 5d–5f. From Figure 5f, it could be found that the hepatocytes recover their normal anatomy, even distribution of sinusoids around the hepatocytes could be observed like control. From Figure 5i and 5l, it could be found that recovery of liver was happened but not the extent as Figure 5f because vacuolated hepatocytes and large sinusoids were still present which indicated that compound **6b** (125 mg/kg) was more effective and safer anticancer agent than compounds **6l** and **6j**. From the histopathological image, it could also be found that compound **6b** (Figure 5f) was as effective as standard drug 5-FU (Figure 5c) in respect to recover the vital organ, liver.

Conclusions

It could be concluded that compound **6b** has shown potent antioxidant activity among all other tested compounds, though other tested compounds have shown moderate to

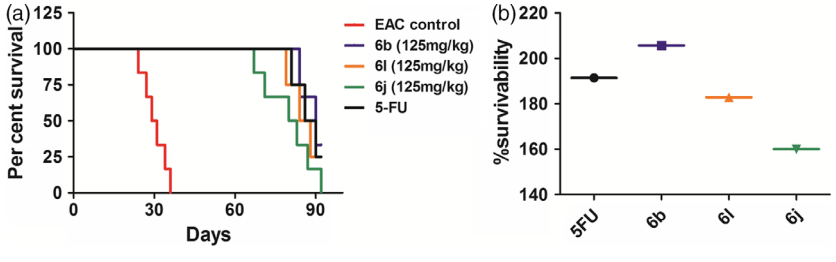

Figure 4 (a) Kaplan–Meir survival plot, (b) comparative study on percent survivability. [Colour figure can be viewed at wileyonlinelibrary.com]

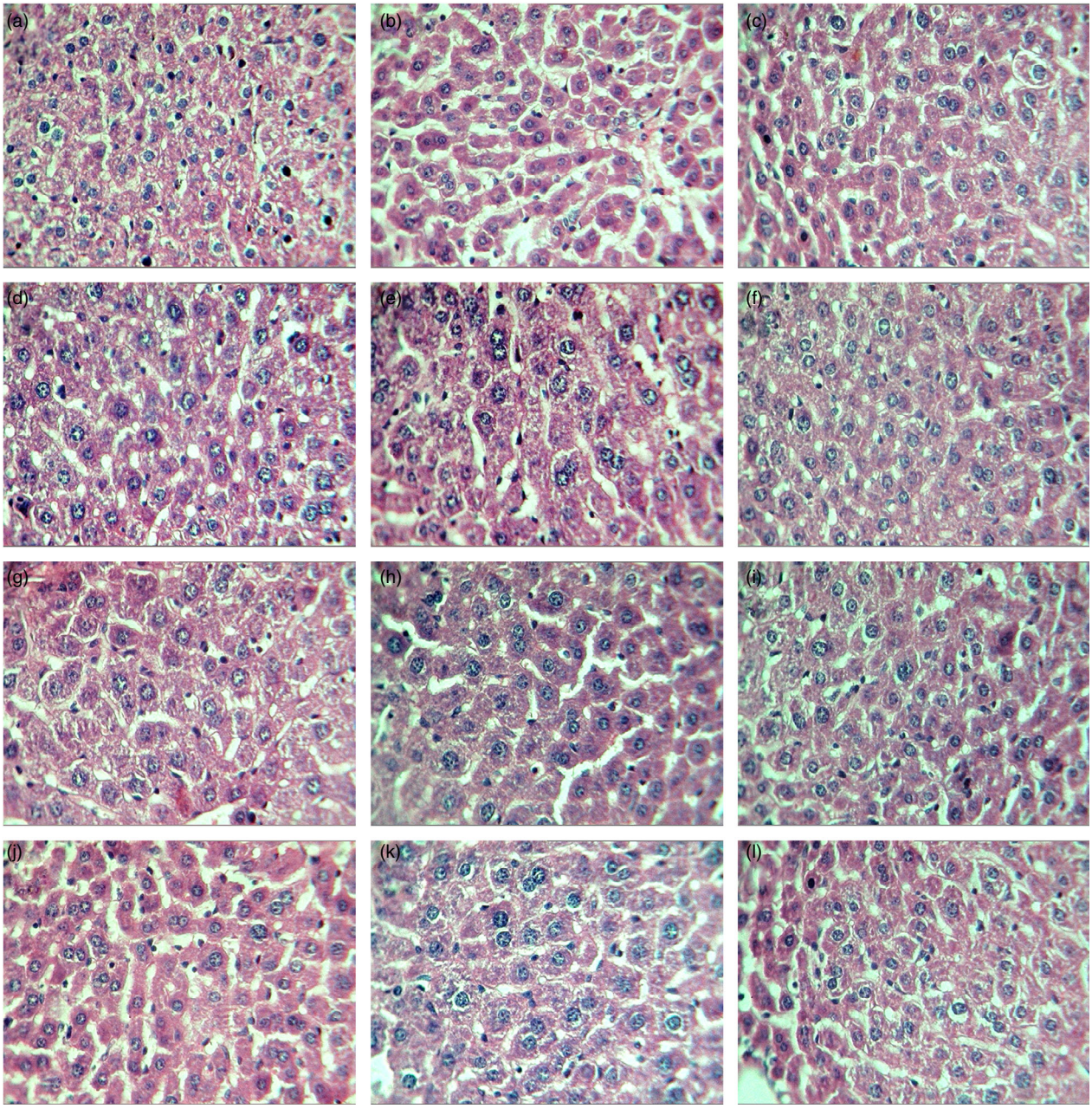

Figure 5 H&E stained liver section of mice; (a) untreated control, (b) Ehrlich ascites carcinoma control, (c) 5-Fluoro Uracil treated (20 mg/kg), (d) treated with 6b (25 mg/kg), (e) treated with **6b** (50 mg/kg), (f) treated with **6b** (125 mg/kg), (g) treated with **6l** (25 mg/kg), (h) treated with **6l** (50 mg/kg), (i) treated with **6l** (125 mg/kg), (j) treated with **6j** (25 mg/kg), (k) treated with **6j** (50 mg/kg), (l) treated with **6j** (125 mg/kg). [Colour figure can be viewed at wileyonlinelibrary.com]

low antioxidant activity compared with the standard, ascorbic acid. It could be suggested from the fact that pyridyl substitution at R^1 position was important to exert superior antioxidant property. On the other hand, the synthesized compounds have shown promising anticancer activity in vitro as well as in vivo. Compounds **6b**, **6j** and **6l** were selected for the in vivo study, and from the results, it could be concluded that compound **6b** was best among other derivatives. The effectiveness could be explained by the addition of pyridyl group at the surface recognition moiety (R^1 position). Molecular mechanism of the therapeutic potency of the synthesized compounds still to investigate. Evaluation of the signalling pathways could be done in future to elicit the anticancer mechanism of the synthesized

compounds. In future, this chemical frame work could also lead to produce compound with more antioxidant and anticancer efficacy with less toxicity.

Declarations

Acknowledgements

Indian Council of Medical Research (ICMR), New Delhi, India was supported this work. Mousumi Das is a Senior Research Fellow of ICMR (No. 45/9/13-PHA/BMS). The authors are very grateful to UGC-UPE Phase – II, Government of India at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata for providing instrumental support.

Conflict of interest

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary information.

Journal of Molecular Structure 1178 (2019) 100–111

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: http://www.elsevier.com/locate/molstruc

Synthesis, characterization, DFT and antimicrobial studies of transition metal ion complexes of a new schiff base ligand, 5-methylpyrazole-3yl-N-(2-hydroxyphenylamine)methyleneimine, (MP_zOAP)

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ARTICLE INFO

Article history:
Received 2 July 2018
Received in revised form
28 September 2018
Accepted 29 September 2018
Available online 4 October 2018

Keywords:
Schiff base
Pyrazole
Metal complexes
DFT
Antimicrobial activities
SEM-study

ABSTRACT

A new pyrazole based 'NNO' tridentate ligand, 5-methylpyrazole-3yl-N-(2-hydroxyphenylamine)methyleneimine, (MP_zOAP) was synthesized and characterized by elemental analyses, mass, IR, ^{1}H NMR spectral parameters. The versatile coordination mode of the ligand was established by the synthesis of Co(III), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) metal ion complexes. These complexes were characterized by elemental analyses, conductance and magnetic susceptibility measurements, UV–Vis, IR, ^{1}H NMR spectroscopy, PXRD and thermal analysis. The structures of the ligand and complexes were also investigated using the DFT method. The ligand structure contains the coordination function of the tertiary nitrogen atom of pyrazole ring, the azomethine nitrogen and the phenolic oxygen atom, suitably spaced for chelation with a metal ion and acting as a 'NNO' tridentate donor ligand. *In vitro* antimicrobial activity of the reported ligand and the metal ion complexes were screened and the mode of action was also studied by scanning electron microscopy (SEM) against some pathogenic bacteria.

1. Introduction

The chemistry of pyrazole containing various types of ligands and their metal ion complexes has attracted considerable interest due to their peculiar coordination chemistry and biological properties [1–11]. There has been considerable progress in biological studies of pyrazole containing Schiff bases, because of the recognition of the importance of the pyrazole moiety in biological processes as antimicrobial [12–16], anti-inflammatory [17–19], antitubercular [20], antitumor [21], anticancer [22], antidepressant [23], anticonvulsant [24], antipyretic [25], anti-angiogenesis [26], anti-parasitic [27], antiviral [28], antibacterial [29], antifungal [30], diuretic and herbicidal activities [31]. They also possess analgesic and anxiolytic activities [32]. It is documented that the presence of fragment –N=CH– in Schiff bases is known for their biological activity [33–35]. There are reports on the structure-activity relationship of this class of compounds [36–39]. Schiff base ligands with nitrogen and oxygen donor set are of particular interest because they provide various coordination modes with metal ions due to lability in structure and sensitivity in molecular environments [40–44]. Condensation reaction of ketone or aldehyde with *o*-amino phenol leading to non-salicylaldehyde based on Salen ligands are elusive in literature and now-a-days are given considerable attention due to their chelating ability with various transition metal complexes by high affinity of phenolate O and neutral N donors giving mono- and poly-nuclear complexes [45–47]. As *o*-amino phenol is a biologically active organic moiety,

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https://doi.org/10.1016/j.molstruc.2018.09.095
0022-2860/

South African Journal of Botany 109 (2017) 16–24

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

A new exploration of *Dregea volubilis* flowers: Focusing on antioxidant and antidiabetic properties

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ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form 30 November 2016
Accepted 13 December 2016
Available online 30 December 2016

Edited by J Van Staden

Keywords:
Dregea volubilis
Antioxidant activity
α-Glucosidase inhibitory activity
α-Amylase inhibitory activity

ABSTRACT

Flowers of *Dregea volubilis* (Family: Apocynaceae) are commonly consumed as seasonal vegetable in India but not yet evaluated for its health beneficiary effects. In the present study, the hydroalcoholic flower extract of *D. volubilis* (DVHA) was evaluated for antioxidant and antidiabetic activities *in vitro*. With high contents of phenolics (39.82 ± 1.22 mg GAE/g) and flavonoids (27.50 ± 0.87 mg QE/g), DVHA showed potential antioxidant activity for scavenging DPPH radical (IC_{50}, 237.86 ± 1.05 μg/mL), hydroxyl radical (IC_{50}, 170.67 ± 0.98 μg/mL), superoxide radical (IC_{50}, 219.07 ± 1.25 μg/mL), nitric oxide radical (IC_{50}, 196.38 ± 1.49 μg/mL) and ferric reducing antioxidant power (176.47 ± 3.18 μmol Fe^{2+}/g), total antioxidant capacity (39.68 ± 1.62 mg GAE/g) along with remarkable inhibitory effects on α-glucosidase (IC_{50}, 3780.09 ± 21.19 μg/mL) and α-amylase (IC_{50}, 360.68 ± 1.26 μg/mL). The characterization of the extract was evaluated by FT-IR and UHPLC analysis. The liquid chromatography study led to the identification and quantification of six compounds viz. gallic acid (412.36 ± 2.29 μg/g), ferulic acid (162.72 ± 0.89 μg/g), rutin (386.25 ± 2.00 μg/g), ellagic acid (208.8 ± 2.00 μg/g), quercetin (306.85 ± 2.24 μg/g) and cinnamic acid (213.71 ± 2.14 μg/g). The results explain the therapeutic potentialities of *D. volubilis* flowers as a potential source of natural antioxidants for use in food and pharmaceutical industries along with their possible applications to control postprandial hyperglycaemia.

1. Introduction

Non-insulin dependent diabetes mellitus, commonly called type 2 diabetes, is a chronic metabolic disease characterized by hyperglycemia which results from insufficient or inefficient insulin secretion. The increasing prevalence of diabetes mellitus has become a major health problem worldwide, reaching epidemic proportions. Controlling post-prandial hyperglycemia through the inhibition of α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) present in the gastro-intestinal tract is one of the major management therapies. To maintain biological processes, oxidation is necessary in living organisms for the production of energy (Shukla et al., 2016). Oxygen derived free radicals and other reactive oxygen species (ROS), which are produced uninterruptedly *in vivo*, are responsible for cell death and tissue damage (Ozsoy et al., 2008). Oxygen radicals have shown to be involved in various diseases like ageing, cancer, cardiovascular diseases, diabetes etc. (Halliwell and Gutteridge, 1999). Antioxidants play an important protective role in cell injury promoted by free radical-induced oxidative stress (Kurutas, 2016). Foods containing natural antioxidants, e.g., polyphenols, which might assist to save living body systems against oxidative damage, have taken a great deal of attention to researchers (Baret et al., 2013; Dey and Lakshmanan, 2013; Hooshmand et al., 2015). Polyphenols have been reported to have antioxidant and hypoglycaemic property with ability to inhibit digestive enzymes such as α-amylase and α-glucosidase (Ang et al., 2015). α-Amylase and α-glucosidase inhibitors can retard utilization of dietary carbohydrates into absorbable monosaccharides and suppress postprandial hyperglycemia, making them applicable for treating type 2 diabetes (Wojdylo et al., 2016). Synthetic enzyme inhibitors used to control postprandial hyperglycemia are undesirable for long term usage because of gastrointestinal side effects and are costly too (Poovitha and Parani, 2016). Natural resources enriched with α-amylase and α-glucosidase inhibitors can be utilized as an effective therapy for treating postprandial hyperglycemia with minimal adverse effects.

Dregea volubilis is a large twining shrub growing in India, Sri Lanka, Myanmar, Indonesia, Thailand, and China (Anonymous, 1976). The plant, commonly known as "Jukti" in Bengali (Nandi et al., 2012),

Abbreviations: DVHA, hydroalcoholic flower extract of *D. volubilis*; GAE, gallic acid equivalent; QE, quercetin equivalent; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; IC_{50}, half maximal inhibitory concentration; FT-IR, Fourier transform infrared; UHPLC, ultra high performance liquid chromatography; ROS, reactive oxygen species; PNPG, *p*-nitrophenyl-α-D-glucopyranoside; TPTZ, 2,4,6 tripyridyl-S-triazine; NBT, nitro blue tetrazolium; NED, N-(1-naphthyl)-ethylenediamine dihydrochloride; DNS, 3, 5-dinitrosalicylic acid; FRAP, ferric reducing antioxidant power; HCl, hydrochloric acid; $FeCl_3.6H_2O$, ferric chloride; $FeSO_4 \cdot 7H_2O$, ferrous sulphate; H_2O_2, hydrogen peroxide; AA, ascorbic acid; TBA, thiobarbituric acid; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; TAC, total antioxidant capacity; TPC, total phenolic content; PBS, phosphate buffered saline.

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http://dx.doi.org/10.1016/j.sajb.2016.12.003
0254-6299/

ChemPubSoc Europe

DOI: 10.1002/slct.201600957

Chemistry SELECT Full Papers

Medicinal Chemistry & Drug Discovery

Promising Antimicrobial Activity of an Oxime Based Palladium(II) Complex

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This work delineates with the development of a novel oxime based palladium complex as a promising antimicrobial agent. A novel oxime based ligand, 3-[(2-hydroxy-naphthalen-1-ylmethylene)-hydrazono]-butan-2-one oxime (**LH**), has been employed to synthesize an orange, monomeric, diamagnetic palladium(II) compound, [Pd(L)(Cl)] (**1**) in moderate yield. **LH** is a mono-condensate Schiff-base of 2,3-butanedionemonoxime monohydrazone and 2-hydroxy-1-naphthaldehyde in tetrahydrofuran. Both the ligand and its palladium compound have been characterized by spectroscopic and analytical techniques. The X-ray crystal structure of **1** reveals that the palladium center in [Pd(L)(Cl)] is nested in a distorted square-planar 'N_2OCl' coordination environment. The electrochemical behavior of [Pd(L)(Cl)] in DMF shows a Pd(II)/ Pd(I) irreversible reduction couple. The in vitro antibacterial activity of [Pd(L)(Cl)] was tested against a good number of human pathogenic Gram-positive and Gram-negative bacterial cell lines. The in vitro antifungal activity of the titled palladium compound was screened against several pathogenic fungal strains. The promising antimicrobial efficacy of the palladium compound was corroborated through SEM study.

Introduction

Development of novel transition metal based compounds for their potential applications as therapeutic[1–3] or diagnostic[3] agents enriches biomedical inorganic chemistry as an integral part of medicinal chemistry.[4] Designing of new ligands through judicious substitution[5,6] and tuning of metal-binding sites[7] offers the opportunity in the modification and advancement of such agents.[8] Medicinal inorganic chemistry[9] has been receiving positive impact over the last few decades due to the promising manifested activity of these therapeutic agents compared to organic-based drugs.[10] Since 1940s, the development of antibacterial drugs and the enhancement of drug resistance capabilities of the bacterial cells towards these newly developed drugs are simultaneously going on.[11] Again, the fungal infections have also been rising alarmingly specifically for immunocompromised people.[12] Patients suffering from critical diseases like AIDS, ailing people taking immunosuppressive drugs for cancer treatment or people who have undergone organ transplantation[13,14] are easily prone to fungal infections. In the last few years, the increasing drug resistance of bacterial[15] and fungal[12] species and the infections caused by them have posed a serious threat to mankind. In this context, metal based drugs are the optimum choice as they might be more bio-specific yet less hazardous to the host. Metal based antibacterial and antifungal agents are benign to environment as well.[16] Palladium(II), a d^8 system, forms a significant number of coordination compounds having structural and thermodynamic resemblance with its congener, platinum(II).[10,17] Schiff-bases, a versatile and flexible type of ligand system, are structurally analogous to natural biological substances.[18] On complexation, their biological activities enhance considerably.[19] Schiff-base complexes are also noteworthy due to the presence of azomethine (C=N) group which interacts with the active sites of the microbial cell constituents.[20] Such active sites often control the possible mode and mechanism of transformation.[18] The excellent and diverse bioactivities of Schiff-base palladium(II) complexes are manifested as antiprotozoal,[1] antibacterial,[21,22] antifungal,[22] antitumor[23,24] and antiviral[24] agents. Polyenes and azoles are two main classes of antifungal organic drugs. Apart from those, sulphur based ligands like dithiocarbamates and their Ni(II), Pd(II) or Pt(II) complexes can also act as fungicides.[16] Again, palladium(II) complexes prepared from nitrogen and sulphur containing ligands like heterocyclic thiolates,[25] thiodiamines[26] or thiosemicarbazides[27] exhibit promising antifungal and/or antibacterial activities. Palladium(II) complexes of only N-donor ligands also show antimicrobial activity.[6] The N- and O-donor palladium complexes were also found to exhibit weak antifungal and antibacterial activities.[11,22] Again, Schiff-bases generated from *ortho*-hydroxyl substituted aromatic aldehydes have been kindling renewed interest in coordination chemistry due to their N- and O-donor sites.[18] Palladium(II) complexes

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/slct.201600957

RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2017, **7**, 29129

Gum odina: an emerging gut modulating approach in colorectal cancer prevention

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It is well established that prebiotics have a profound influence on colonic microbiota which in turn play an essential role in ameliorating the host's health. This study is focused on Gum Odina (GO), a reported prebiotic in our earlier work, and its impact on colorectal cancer (CRC). GO, upon utilization by probiotics, liberates short-chain fatty acids, acetic acid (0.864 ± 0.050 mg ml^{-1}) and butyric acid (2.303 ± 0.083 mg ml^{-1}) predominantly and increases colonization of *Lactobacillus* sp. and *Bifidobacterium* sp. in a gut simulator. The *in vivo* preventive study of CRC was conducted on Swiss albino mice using 1,2-dimethyl hydrazine (DMH) along with inulin and GO as the standard and test prebiotic, respectively. Scanning electron micrographs of the colon depict that the severity of mucosal dysplasia, flat lesions and loss of goblet cells was quite low in the GO group compared to the DMH alone treated group. The same was noticed in the histomicrograph in terms of alteration of the colonic architecture and abnormalities in the submucosa. Administration of DMH also caused oxidative burst as the levels of reactive oxygen species and lipid peroxidation significantly increased ($p < 0.05$) but reduced by 29.35% and 27.65%, respectively, in the GO group. Moreover, the levels of glutathione, glutathione-*S*-transferase, superoxide dismutase and catalase in the colonic tissues significantly increased ($p < 0.05$) by 31.26%, 10.96%, 12.4% and 6.37%, respectively, when compared to IN, a standard prebiotic. Thus, GO possesses CRC-preventing along with antioxidant properties and slows the overall tumor genesis process.

Received 10th April 2017
Accepted 18th May 2017

DOI: 10.1039/c7ra04077f

rsc.li/rsc-advances

1. Introduction

The use of prebiotics for an effective gut ecology is a current trend of research,[1] as the demand in natural products claiming health benefits is increasing day to day. This developing aspect of glycoscience is coming into the limelight due to increased health consciousness and consumer awareness[2] leading to the development of new bioactive compounds working on the principle of "prevention is better than a cure".[3]

Prebiotics are carbohydrates[4] that resist digestion and absorption in the upper parts of the gastrointestinal tract[5] of the host, selectively utilizing specific bacterial strains,[6] and confer health benefits.[7] Considering the profound health benefits of prebiotics, their development from new natural sources like Gum Odina (GO),[1] mushroom polysaccharide,[8] *Aloe vera* mucilage,[9] artichoke fibers (*Cynara cardunculus* L. var. scolymus),[10] almond skin,[11] bamboo shoots[12] (*Phyllostachys praecox*) and *Stevia rebaudiana* (Bertoni) roots[13] is being carried out. Prebiotics not only prevent gastrointestinal diseases[14] by limiting the space of survival for pathogenic bacteria[15] but also improve human health by positive stimulation of the immune system,[16] reduction of intestinal inflammatory diseases and cholesterol levels,[14] regulation of blood glucose,[17] and treatment of pochutis[18] and osteoporosis.[2]

Due to recent occupational hazards and dietary habits like high red meat intake, colorectal cancer (CRC) has become the most deadly cancer in the world at present.[18] CRC is mainly caused by mutation in tumor suppression genes[19] and prebiotics act as a chemopreventive agent by removing food-borne mutagens.[20] Pathogenic bacteria, upon colonization in the intestine, contribute to progression of CRC by inducing gut inflammation,[21] up-regulating inflammatory genes (NF-kB, IL-6, IL-8 and IL-18),[22] epithelial damage and promoting pro-oncogenic responses.[23] Prebiotics, conversely, manipulate gut microbiota[24] *i.e.* selectively stimulate *Lactobacilli* sp. and *Bifidobacterium* sp. immunomodulation[25] by increasing sIgA in the gut environment,[1] enhance apoptosis,[26] and down-regulate the expression levels of COX-2, NF-kB and iNOS.[27] Moreover, upon utilization, prebiotics also liberate SCFA[28] which slows the overall tumerigenesis[29] by providing energy to colonocytes.

GO has been used previously in formulations like tablet binders,[30] emulsifying agents[31] and matrices for sustained drug release,[32] and in designing chitosan–GO complex coacervates for colon targeted drug delivery.[33] The chemical composition[34] and branched structure[35] of this polysaccharide is reported

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RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2016, **6**, 106563

Antifungal ouzo nanoparticles from guar gum propionate†

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Received 3rd August 2016
Accepted 25th October 2016

DOI: 10.1039/c6ra19658f

www.rsc.org/advances

Herein, we report the synthesis of a guar gum propionate ester and the self-assembly formation of nanoparticles following a facile solvent shifting technique. Hofmeister-ion-guided homogeneous phase reactions were applied in guar galactomannan chemistry. A high degree of substitution (DS) was achieved due to an enhanced access to surface hydroxyl groups in the presence of chaotropic Hofmeister ions. The resulting biopolymer was fully characterized using FT-IR, ^{13}C NMR, XRD, and thermal analysis. SEM and TEM studies confirmed the formation of quasi-spherical self-assembly nanoparticles in water. The nanoparticles expressed widespread antifungal activities due to the particles' dynamics and the presence of surface propionate moieties. The minimum fungicidal concentration (MFC) in *Cryptococcus neoformans* and *Candida albicans* were recorded at 500 μg mL^{-1}. The development of guar gum propionate self-assembly nanoparticles has great potential in biomedical device applications.

1. Introduction

Fungus infections in both the plant and animal kingdom are a serious concern in the present day. The widespread and extensive use of fungicides has given rise to drug resistance. The propagation of opportunistic fungal infections in humans is also very common.[1,2] On the contrary, antifungal compounds are scarce and are often very toxic. Safer agents for fungus control are therefore very important in different application areas, such as with sanitary materials, medical devices, food security, and agriculture. Fast growing interest therefore exists for alternative fungus growth control techniques.[3] Metal oxides and metal nanoparticles were investigated earlier,[4] and in one report borneol-grafted cellulose was studied as an effective fungicide.[5] The environmental stability and biosafety of metal nanoparticles and cellulose derivatives, however, are generally unacceptable, whereas biopolymer nanoparticles offer safer tools for the effective control of prokaryotic growth and propagation.[6] Additionally, nanoscale assemblies express greater surface functionality than the bulk. This effect was thus considered for enhanced fungus membrane interactions, nanoparticle affinity, and killing *via* membrane damage.

Guar gum (GG) is a high molecular weight hydrated biopolymer obtained directly from the *Cyamopsis tetragonoloba* seed pericarb. The biopolymer presents a very ordered structure of a β 1–4 mannose chain interposed with α 1–6 galactose substituents in almost every second unit.[7] GG is used regularly as a functional food, in cosmetics, ice creams, and in drug delivery.[8,9] The biopolymer can be functionalized at the surface OH groups following nucleophilic substitution, or *via* etherification or esterification reactions.[10] Different techniques have been adopted for GG ethers, esters, and click chemistry products syntheses.[11–13] Alkali-catalyzed substitution products, like carboxymethyl guar gum and hydroxypropyl guar gum, are popular in petroleum drilling applications.[14,15] Some other covalently modified GGs were explored in different specialized applications.[11,16] Most GG derivatives developed so far, however, permit huge water penetration and the products are vulnerable due to ion-mediated hydrolysis and microbial decomposition. In one occasion, GG benzoates were reported as water retardant films for bacterial killing in a water environment.[17]

In the present study we used a chaotropic Hofmeister-ion-mediated homogeneous phase esterification reaction to develop a water insoluble guar gum propionate derivative (GGP). A higher degree of substitution (DS) was achieved and the product could solubilize easily in DMSO. Surfactant-free water dispersible GGP nanoparticles were further developed using a facile solvent shifting 'ouzo effect' technique. Propionates are safe agents for microbial control in food and beverages.[18] Propionate salts are, however, ineffective in lipid water interfaces. Nearly 8% to 12% of the propionic acid by mass is required for effective mold control.[19] High-mobility self-

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ra19658f

Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2016, **7**, 3064

Prebiotic potential of gum odina and its impact on gut ecology: *in vitro* and *in vivo* assessments

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Received 18th December 2015,
Accepted 11th May 2016

DOI: 10.1039/c5fo01581b

www.rsc.org/foodfunction

The use of prebiotics to escalate certain gut flora is a current aspect of research for effective gut ecology. In the present study we appraise the efficacy of gum odina obtained from the bark of *Odina wodier* (Anacardiaceae), which is not fully degraded (16%) in the upper GI tract and becomes available to the lower region, as a prebiotic. An *in vitro* prebiotic activity assay established a quantitative score to describe the extent to which gum odina supports the selective growth of probiotics with a maximum of 5.60 ± 0.11 for *Lactobacillus plantarum* MTCC 6160. The polysaccharide, upon fermentation, also liberates lactic acid (0.46 ± 0.003 mg ml^{-1}) and acetic acid (1.03 ± 0.003 mg ml^{-1}). *In vivo* studies revealed that natural gum selectively stimulates *Lactobacillus* sp., and eliminates enteric pathogens with a C.F.U. of 384.48 ± 0.11 and 40.56 ± 0.17 respectively on the 8^{th} day. The changes in the level of β-galactosidase signify maturation of macrophages in the gut environment. It also boosts the immune system by increasing sIgA upon infection from the 5^{th} day in the gut, when incorporated into the feed of mice. Moreover an increase in levels of IFNγ on the 5^{th} day also manifest additional protection against various pathogen-induced primary and secondary infections. Thus, gum odina is a potential prebiotic which not only provides nutrition but also improves gut ecology.

1. Introduction

In response to an infection, dehydration or to tackle unfavorable conditions, many trees and shrubs produce an aqueous thick exudation[1] triggered by changes in the existing cell wall by a process known as Gummosis.[2] The solid exudation is referred to as natural gum.[1] Gum odina is extracted from the tree *Odina woodier*, Roxb, Family Anacardiaceae.[3] It is a mixture of various polysaccharides[4] and chemically is a negatively charged polyelectrolyte, belonging to the glycuronogalactan polysaccharides.[5,6] The structure of gum odina was reported earlier[7] which is a polymer of 63.70% D-galactose, 19.50% L-arabinose and 11.50%–17% of two uronic acids (D-galactouronic acid and aldobiuronic acid). The molecular weight of natural gum was reported to be 1.68×10^5 as determined by the static light scattering method.[8] It consists of a 1,6 β-D-galactopyranosyl residue as revealed from compositional analysis with slightly branched galactopyrose units as side chains linked through C_1, C_3, and C_6. Gum odina is chemically similar to inulin *i.e.* the majority of the glycoside linkages are of β-type.[9] Research in the past has revealed the ability of gum to act as a tablet binder[3] and currently the natural gum is used for sustained release drug delivery systems.[10]

Of late the development of prebiotics is another trending area of research.[11] Prebiotics are usually carbohydrates,[12] which are not digested in the stomach by the host, and stimulate growth and activity of specific beneficial strains of bacteria in the gut over others,[13,14] referred to as probiotics[15] in the colon. A large variety of microbial flora is present in human body throughout the gut depending on various physicochemical conditions.[16] Among various resident intestinal bacteria lactobacilli and bifidobacteria are stimulated by prebiotics.[15] Prebiotics upon metabolization[17] liberate lactic, acetic and other short chain organic acids possessing anticancer properties and also provide nutrition to probiotics. Thus synbiotics or a combination of probiotics and prebiotics have a more beneficial effect on the gut than probiotics or prebiotics alone.[15] Synbiotics also eliminate harmful and pathogenic microbes[18] by limiting the space for survival. The association of prebiotics with probiotics has the ability to influence and improve the health of humans.[19] Most of the commercially available prebiotics are generally fructo-oligosaccharide (FOS)[20] like inulin, glacto-oligosaccharides (GOS),[21] arabinoxylans,[22] xylooligosaccharides,[23] pectic-oligosaccharides[24] and galactoglucomannans[25] which are generally plant polysaccharides. Prebiotic research targeted on distal colonic

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European Journal of Medicinal Chemistry 89 (2015) 59–66

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, structure, spectral characterization, electrochemistry and evaluation of antibacterial potentiality of a novel oxime-based palladium(II) compound

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ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form
3 September 2014
Accepted 12 October 2014
Available online 14 October 2014

Keywords:
Oxime
Palladium
Crystal structure
Redox
Antibacterial
SEM study

ABSTRACT

The title monomeric Pd(II) compound, [Pd(L)(Cl)], was synthesized in moderate yield out of the reaction of equimolar proportion of $Na_2[PdCl_4]$ and 3-[(5-bromo-2-hydroxy-benzylidene)-hydrazono]-butan-2-one oxime (**LH**) in tetrahydrofuran milieu. **LH** is a 1:1 Schiff-base condensate of 2,3-butanedionemonoxime monohydrazone and 5-bromosalicylaldehyde. [Pd(L)(Cl)] has been characterized by C, H and N microanalyses, ^1H and ^{13}C NMR, FAB-MS, FT-IR, Raman spectra, UV–Vis spectra and molar electrical conductivity measurements. [Pd(L)(Cl)] is diamagnetic. Structural elucidation reveals that the palladium center in [Pd(L)(Cl)] is nested in 'N_2OCl' coordination environment. The geometry around Pd in [Pd(L)(Cl)] is distorted square-planar. The redox behavior of [Pd(L)(Cl)] in DMF shows a reduction couple, Pd(II)/Pd(I) at −0.836 V versus Ag/AgCl. The *in vitro* antimicrobial activity of [Pd(L)(Cl)] was screened against both Gram-positive and Gram-negative human pathogenic bacteria. This bioactivity was substantiated with SEM study. [Pd(L)(Cl)] exhibits satisfactory bactericidal as well as bacteriostatic activity.

1. Introduction

Synthesis, characterization and evaluation of biological activities of novel metal-based compounds are a rapidly growing area of contemporary research [1–3]. Drugs based on transition metal compounds are superior to organic-based drugs so much so that medicinal inorganic chemistry now is an emerging area of research [4]. Again, bacterial drug resistance potentiality is increasing alarmingly [5]. Consequently, development of new therapeutic agents to combat bacterial infection warrants current attention [6,7]. Synthesis of novel antibacterial drugs with properly known structure is important to understand their behavior in biological environment and subsequent possible mode of action. The transition metal compounds of hetero donor ligands show promising biological activities with reduced toxicity [8]. Palladium, an important platinum group metal [9], is noteworthy in this perspective. The compounds of palladium manifest diverse biological activities [10]. Palladium-based compounds are well-known antitumor [11], antiviral [12], antifungal [13], antiinflammatory [13], antimalarial [14] and antibacterial [15] agents. The intense and diverse bioactivities of various palladium compounds on bacterial strains are well-documented in the literature [10,15,16]. Pd(II) being a soft Lewis acid has an inherent tendency to bind soft donor centers like N, S in its d^8 square-planar structural disposition [17]. Accordingly, most of the antibacterial palladium(II) compounds are from N- and S-based ligands like thiosemicarbazones [16–18], thiocarbazates-thioamides [10], thiodiamines [19] etc. Palladium(II) compounds, synthesized from ligands with N donor only, are also known to exhibit antimicrobial activities against Gram-negative strains like *E. coli*, *P. aeruginosa*, *S. marcescens* along with Gram-positive strains like *S. aureus*, *B. subtilis* [20]. Thus it seems that for showing bioactivity, palladium complexes of N, S donor ligands are of obvious choice. Examples are also known from ligands having N, O donor ligands [21]. Palladium complexes from the alkyl derivative of thiosalicylic acids having S, O donor sides

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http://dx.doi.org/10.1016/j.ejmech.2014.10.035
0223-5234/

Med Chem Res (2015) 24:1952–1963
DOI 10.1007/s00044-014-1261-0

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Bioassay directed isolation of a novel anti-inflammatory cerebroside from the leaves of *Aerva sanguinolenta*

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Received: 5 July 2014 / Accepted: 11 September 2014 / Published online: 27 September 2014

Abstract This paper attempts to evaluate the anti-inflammatory potential and the possible mechanism of action of the leaf extracts and isolated compound(s) of *Aerva sanguinolenta* (Amaranthaceae), traditionally used in ailments related to inflammation. The anti-inflammatory activity of ethanol extract (ASE) was evaluated by acute, subacute and chronic models of inflammation, while a new cerebroside ('trans', ASE-1), isolated from the bioactive ASE and characterized spectroscopically, was tested by carrageenan-induced mouse paw oedema and protein exudation model. To understand the underlying mechanism, we measured the release of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin (PG)E2, along with the cytokines like tumour necrosis factor (TNF)-α, and interleukins(IL)-1*β*, IL-6 and IL-12 from lipopolysaccharide (LPS)-stimulated peritoneal macrophages. The results revealed that ASE at 400 mg/kg caused significant reduction of rat paw oedema, granuloma and exudative inflammation, while the inhibition of mouse paw oedema and exudative inflammation by ASE-1 (20 mg/kg) was comparable to that of the standard drug indomethacin (10 mg/kg). Interestingly, both ASE and ASE-1 showed significant inhibition of the expressions of iNOS2 and COX-2, and the down-regulation of the expressions of IL-1β, IL-6, IL-12 and TNF-α, in LPS-stimulated macrophages, via the inhibition of COX-2-mediated PGE2 release. Thus, our results validated the traditional use of *A. sanguinolenta* leaves in inflammation management.

Keywords *Aerva sanguinolenta* · Cerebroside · Anti-inflammatory · COX-2 · PGE2

Electronic supplementary material The online version of this article (doi:10.1007/s00044-014-1261-0) contains supplementary material, which is available to authorized users.

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Introduction

Inflammation is a complex biological response of vascular tissues to harmful stimuli including a protective attempt to remove the stimuli and initiate the healing process, and has been classified as acute or chronic. Acute inflammation, the initial response of the body to the harmful stimuli, takes place through the increased movement of plasma and granulocytes from blood to the injured tissues (Colditz, 1985). A cascade of biochemical events involving the vascular and immune systems and various cells of the injured tissues propagate and mature the response (Kasama *et al.*, 1993). Simultaneously various inflammatory mediators including bioactive lipids, reactive oxygen and nitrogen species, cyclooxygenase (COX)-2 and pro-inflammatory

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 7, Issue 3, 2014

ISSN - 0974-2441

Original Article

DETERMINATION OF EFFICACY OF A NATURAL TABLET BINDER: CHARACTERIZATION AND *IN- VITRO* RELEASE STUDY

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Received: 16 May 2014, Revised and Accepted: 8 June 2014

ABSTRACT

Objective: The objective of the study concerns the evaluation of gum Odina as a novel pharmaceutical aid for the development of tablet formulation.
Methods: The tablet weight (850mg) and thickness (8mm) was kept constant. Paracetamol was used as reference drug. Wet granulation technique was used for the preparation of Paracetamol granules. The binder concentrations used in the formulation were 0.125%, 0.250%, and 0.375%. The prepared powder mixtures were subjected to both pre and post compression evaluation parameters including; IR spectroscopy, Micromeritics, tablet hardness, friability, disintegration time and *in-vitro* drug release. Compatibility of the drug with the gum was studied using FTIR.
Results: The results of micromeritics studies revealed that all formulations were good flowability. Tablet hardness and friability indicated good mechanical strength. *In vitro* dissolution studies indicated that the release of drug from tablet with 0.125% gum odina was 98.55% in 30 minute but release was delayed with 0.25% and 0.375% gum odina.
Conclusion: It is concluded that the gum odina requires less amount as a tablet binder than starch with complying all parameters.

Keywords: Gum Odina, Tablet binder, Paracetamol, Wet granulation, *In-vitro* drug release.

INTRODUCTION

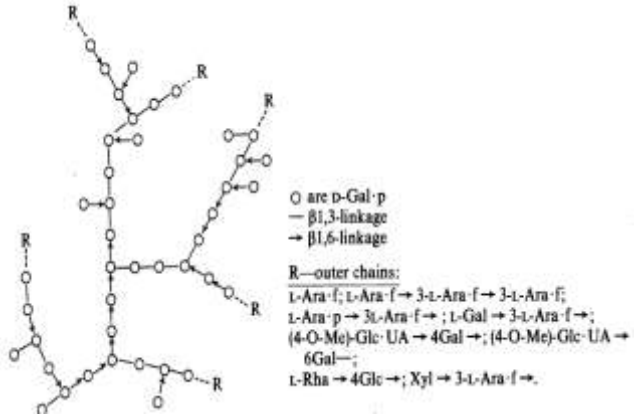
As a natural defense mechanism to prevent infection or dehydration many trees and shrubs are known to produce an aqueous thick exudation and the solution dries up in contact with sunlight and air and a hard transparent brown-tint glass like mass is formed. This solid exudation is commonly known as natural gum [1, 2]. Gum Odina is a natural gum obtained from *Odina wodier*, Roxb. Family Anacardiaceae which is found in deciduous forest of India. Moreover the plant is also found in Myanmar, Srilanka, China, Malaysia, Cambodia and Philippine Island [3]. Natural gum is normally neutral or slightly acidic complex of polysaccharides or partially acetylated polysaccharide or heterogeneous polysaccharide obtained as a mixture of calcium, potassium and magnesium salts [4, 5].

Recently Sinha et al., 2011 has evaluated the efficacies of gum matrices for control release. Previously in 2006 we have evaluated only the binding capability of the gum by comparing it with the standard starch paste as a tablet binder. In that study it was demonstrated that the gum provides desired hardness, binding and disintegration time to the formulation [6]. In present work we studied the release kinetic by taking a model drug along with other parameters like micromeritics, gum characteristics and statistical analysis etc. This is the further continuation of research work to evaluate the efficacy of gum in so many aspects.

Brief Chemistry of Gum Odina

Gum odina is a negatively charged polyelectrolyte, belonging to the glycuronogalactan polysaccharides [7] and the proposed structure of glycuronogalactans as suggested by Bhattacharya and Rao, 1964 is given below.

To evaluate the efficacy of Gum Odina as a tablet binder and the potential binding capability of the gum has been evaluated with the standard starch paste as a tablet binder. The objective of the study is to establish the potential of gum Odina as a novel pharmaceutical aid for development of instant release Paracetamol tablet. The influence of varying the proportion of the gum, the nature of diluents and their ratio in the preparation was also evaluated. Compatibility of the drugs with the gum was studied using FTIR. *In vitro* dissolution studies indicated that various proportions of Odina gum having different release pattern. As the natural materials is readily available, cost effective, eco-friendly, potentially degradable and compatible due to its origin it can be used in the field of drug formulation in near future.



MATERIALS AND METHODS

Materials

Paracetamol was obtained as a gift sample from Alkem Laboratories Ltd, Himachalpradesh. Ethanol (Jiangsu Huaxi International Trade Co Ltd, China). Microcrystalline cellulose, starch, talc purified, silicon dioxide and magnesium stearate (E.Merck Ltd, Mumbai). All the other chemicals and solvents used for the study were of analytical grade and used without further purification. RO water was used for preparation of dissolution medium.

Collection of gum

Gum was collected from the tree *Odina wodier*, Roxb. family Anacardiaceae during Autumn in the month of August from the MandalGhat of Jalpaiguri District, West Bengal, India. The gum was the natural exudates on the bark of the tree and it was collected in dry condition. The plant was identified by Dr. R.P. Nandi, Director, Cinchona, Mangpoo, Darjeeling, West Bengal, India and the voucher specimen has been kept with the Director of Cinchona, Mangpoo[6].

Polyhedron 68 (2014) 122–130

Contents lists available at ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

Synthesis, characterization, X-ray crystallography and antimicrobial activities of new Co(III) and Cu(II) complexes with a pyrazole based Schiff base ligand

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ARTICLE INFO

Article history:
Received 30 June 2013
Accepted 5 October 2013
Available online 23 October 2013

Keywords:
Schiff base ligand
Co(III)
Cu(II)
Crystal structure
Antimicrobial activities

ABSTRACT

A new pyrazole based 'NNS' tridentate ligand, 5-methylpyrazole-3yl-N-(2′-methylthiophenyl)methyleneimine (MPzOATA), has been synthesized and characterized by elemental analyses, mass, IR and NMR spectroscopy. The versatile coordination mode of the ligand has been established with the synthesis of two metal–organic complexes with different metal ions, Co(III) (complex **I**) and Cu(II) (complex **II**). These metal–organic complexes have been characterized by single crystal X-ray structure analyses, along with several spectral techniques. Structural analyses of both the monomeric complexes reveal that complex **I** crystallized in the monoclinic *C*2/*c* space group, while complex **II** crystallized in the orthorhombic Pbca (#61) space group. In complex **I**, the Co(III) centre shows a six coordinate octahedral geometry, while in complex **II**, the Cu(II) centre shows a five coordinate square pyramidal geometry. The reported ligand and the metal ion complexes have been screened for their in vitro antimicrobial activity against some pathogenic bacteria.

1. Introduction

Schiff base ligands containing nitrogen and sulfur atoms as donor atoms and their metal complexes have attracted considerable interest in chemistry and biology owing to their structural features and potentially beneficial biological activities, which have often been related to a chelation phenomenon with trace metal ions [1–3]. The chelating nitrogen and sulfur atoms can stabilize unusual oxidation states and uncommon coordination numbers in metal ion complexes [4]. Transition metal ion complexes of nitrogen-sulfur donor chelating ligands are active centers of some oxidoreductases, such as hydrogenase, xanthine oxidase and nitrogenase [5–7]. The five-membered N-heterocycle pyrazole (1:2 diazole), being isomeric with imidazole (1:3 diazole), an integral part of the well-known histidine residue of many proteins, has attracted considerable attention as a coordinating ligand for complex formation with biologically important metal ions like Fe(III), Co(III), Ni(II) and Cu(II) and for its outstanding role in medicinal chemistry as an anti microbial, anti toxic, anti viral, ACE (angiotensin-converting-enzyme) inhibition and anti-inflammatory agent [8–13]. Pyrazoles are π-excessive, better π-donors and weaker π-acceptors than six membered heterocycles and can act as a hard donor site [14,15]. The pyrazole nucleus is very stable, thermally and hydrolytically, and can act as a monodentate or bidentate ligand by deprotonation of the N(1)–H group [16,17].

In this endeavor, our efforts have focused on the design and synthesis of a new pyrazole based "NNS' tridentate Schiff base ligand, 5-methylpyrazole-3yl-N-(2′-methylthiophenyl)methyleneimine (MPzOATA). Two different metal–organic complexes, {[Co(MPzOATA)$_2$]PF$_6$} (complex **I**) and [Cu(MPzOATA)Cl$_2$] (complex **II**), have been synthesized with the ligand and characterized by single crystal structural analysis and other spectral studies. The synthesized compounds have been screened for antimicrobial activities against some pathogenic bacterial strains.

2. Experimental

Mueller-Hilton Agar was purchased from Merck India Ltd. All other reagents were of AR grade/molecular biology grade and were obtained from commercial sources and used without further

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0277-5387/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.poly.2013.10.016

Journal of Coordination Chemistry, 2014
Vol. 67, No. 2, 286–299, http://dx.doi.org/10.1080/00958972.2014.880426

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Synthesis, characterization, X-ray crystallography, and antimicrobial activities of Ni(II) and Cu(II) complexes with a salicylaldehyde-based thiosemicarbazone ligand

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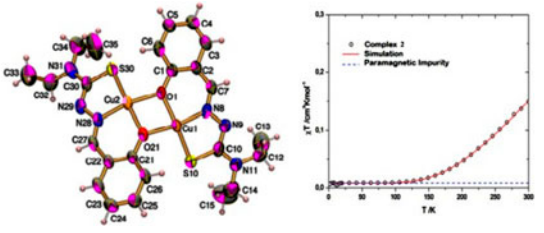
(*Received 9 October 2013; accepted 5 December 2013*)



A new salicylaldehyde-based 'ONS' tridentate salicylaldehyde-N(4)-diethylthiosemicarbazone (H_2SANEt_2) has been synthesized and characterized by elemental analyses, mass, IR, and ^1H NMR spectral parameters. The coordination mode of the synthesized ligand is reported by solid state isolation and physico-chemical identification of Ni(II) and Cu(II) complexes, $[Ni(SANEt_2)]_2$ (**1**) and $[Cu(SANEt_2)]_2$ (**2**). Both complexes are neutral and oxygen-bridged dinuclear species. The ligand is bideprotonated 'ONS' tridentate in both complexes. IR spectral data indicate that coordination of each metal of the two complexes occurs through phenolic oxygen, azomethine nitrogen, and thiolato sulfur. X-ray crystallographic data of **1** and **2** (both monoclinic, $P2_1/c$) show a MONSO (where M=Ni and Cu) square-planar coordination around each metal. Magnetic studies of **2** show that the two Cu(II) centers are strongly antiferromagnetically coupled via the bridging phenoxo O. All the synthesized compounds were tested for their *in vitro* antimicrobial activities against pathogenic bacteria. The inhibition power of the complexes was greater than the parent ligand.

Keywords: Thiosemicarbazone; Ni, Cu-complexes; Crystal structures; Magnetic property; Antimicrobial activity

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Polyhedron 56 (2013) 211–220

Contents lists available at SciVerse ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

A palladium(II) complex: Synthesis, structure, characterization, electrochemical behavior, thermal aspects, BVS calculation and antimicrobial activity

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ARTICLE INFO

Article history:
Received 9 January 2013
Accepted 26 March 2013
Available online 10 April 2013

Keywords:
Oxime
Palladium
Structure
BVS
Redox
Cytotoxicity

ABSTRACT

The reaction of equimolar proportions of $Na_2[PdCl_4]$ and 1-(4-methylimidazol-5-yl) phenylhydrazonopropane-2-one oxime (LH), a 1:1 Schiff-base condensate of 1-hydrazono-1-phenyl-propan-2-one oxime (**1**) and 4-methylimidazole-5-carboxaldehyde, in methanol gives rise to [Pd(L)(Cl)] (**2**) in a satisfactory yield. The title monomeric palladium(II) complex, **2**, has been characterized by C, H and N microanalyses, ^1H and ^{13}C NMR, FAB-MS, FT-IR, UV–Vis spectra, molar electric conductivity measurements and room temperature magnetic susceptibility measurements. The X-ray crystal structures of **1** and **2** have been determined. The structure of **1**, a precursor of the ligand (LH), shows that the methyl and phenyl groups are in an '*anti*' disposition. Compound **1** crystallizes in the monoclinic space group $P2_1$ with $a = 6.9503(5)$, $b = 6.4535(3)$, $c = 10.1631(6)$ Å, $V = 455.04(5)$ Å^3 and $Z = 2$. The structure of **2** reveals that it is a distorted square-planar palladium(II) compound. The palladium center is in an 'N_3Cl' coordination chromophore. Complex **2** crystallizes in the tetragonal space group $I4_1/a$ with $a = 22.5744(3)$, $b = 22.5744(3)$, $c = 13.2967(2)$ Å, $V = 6776.05(16)$ Å^3 and $Z = 16$. The thermal and electrochemical aspects of **2** have been studied. Electrochemical studies in DMF show a Pd(II) to Pd(III) oxidation at 0.573 V, along with a reduction of Pd(II) to Pd(I) at −0.757 V versus Ag/AgCl. A Bond-Valence Sum (BVS) model calculation was performed to assign the oxidation state of the palladium center in **2**. The *in vitro* antimicrobial activity of **2** was tested against both Gram positive and Gram negative bacteria. Complex **2** exhibits satisfactory bacteriostatic activity.

1. Introduction

In terms of annual demand, palladium is one of the most important platinum group metals [1]. Pd(II) complexes have attracted renewed interest due to their wide-spread applications in organic syntheses [2], catalytic processes [3], biological and pharmacological usage [4]. The palladium catalyzed Suzuki–Miyaura cross-coupling reaction represents one of the most widely used processes for the synthesis of biaryls [5–8]. In addition, palladium-catalyzed Heck reactions of aryl halides with alkenes have also become one of the most powerful tools in organic synthesis for the making of the carbon–carbon bond [9,10]. The use of Pd-catalysts in Suzuki–Miyaura and Heck-Mizoroki cross-coupling reactions in aqueous media stems interest from a viewpoint of green sustainable chemistry [11,12]. Apart from catalytic aspects, Pd(II) compounds have inherent cytotoxic behavior. On the basis of the structural analogy (d^8 ions in a square-planar geometry) and the thermodynamic difference with Pt(II) complexes, there is much interest in the study of palladium(II) complexes as potential anticancer drugs, especially those bearing the chelating ligands [13–15]. In this context it is interesting to note that the first monomeric Pd(II) complex having excellent antiproliferative activity, as demonstrated by *in vitro* experiments, was a mixed ligand Pd(II) complex [16]. The associated ligand was, as usual, a thiosemicarbazone based ligand with triphenyl phosphine as an ancillary ligand [17,18]. Curiously such type of bioactivity of a monomeric Pd(II) compound with an N,O-donor oxime-based ligand is unprecedented. Herein we wish to report the synthesis, characterization, structure, thermal and redox behavior of a monomeric Pd(II) complex synthesized from an oxime-based ligand. The antimicrobial activity of the ligand, 1-(4-methylimidazol-5-yl) phenylhydrazonopropane-2-one oxime (LH), and its palladium(II) compound, **2** was investigated against

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0277-5387/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.poly.2013.03.064

INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com ISSN 2230 – 8407

Research Article

ANTICANCER POTENTIAL OF METHANOLIC AND AQUEOUS EXTRACT OF *LEUCAS INDICA* (LINN.) AGAINST EHRLICH ASCITES CARCINOMA CELLS ON SWISS ALBINO MICE

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Article Received on: 18/03/13 Revised on: 01/04/13 Approved for publication: 21/05/13

DOI: 10.7897/2230-8407.04641

IRJP is an official publication of Moksha Publishing House. Website: www.mokshaph.com

ABSTRACT

The present study was designed to evaluate the *in vivo* anticancer activity of methanolic and aqueous extract of *Leucas indica* Linn. (Aerial parts) against Ehrlich's Ascites Carcinoma (EAC) on Swiss albino mice. The anticancer activity was done by intra peritoneal transplantation of EAC cells into mice and subsequently analysis of ascitic fluid, haematological, biochemical and histopathological parameters along with body weight and percentage of life span. The animals were treated with the extracts at the dose level of 200 and 400 mg/kg, intra peritoneally and compared to the reference drug vinblastine. Both the extracts showed significant dose dependent reduction of viable cells (cancer cells), increased non-viable cells (dead cells), decreased in body weight and increased the duration of life span. In case of hematological study the extracts showed increased hemoglobin level and RBC count but decreased WBC count. The certain important serum enzymes like glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and alkaline phosphatase levels were also decreased in treated group of animals. Finally, in case of histopathological investigation of livers showed a significant protection and regeneration of EAC induced oxidative stress and cellular damage. The preliminary phytochemical screening of the extracts revealed the presence of flavonoids, total phenolic compounds, saponin and tannin. However the aqueous fraction of the *Leucas indica* possesses more potential anticancer property compared to methanolic fraction. The anticancer property of this plant was considered due to the presence of flavonoids and total phenolic compounds.

Keywords: *Leucas indica*, Methanolic extract, Aqueous extract, Anticancer, EAC cell, Vinblastine.

INTRODUCTION

Cancer is the second life threatening disease next to cardiovascular disorders, characterized by uncontrollable cellular growth, local tissue invasion and distance metastasis and cause more than 6million deaths per year and may increase up to 11.5 millions in the year of 2030 world wide[1,2]. To combat this so far mortal disease currently there are mainly three types of modern treatment like chemotherapy, radiotherapy and surgical procedure are available. Most of the chemotherapeutic agents cause severe toxicity on bone marrow cell, epithelial tissue, reticuloendothelial system, gonads etc. due to their high potency and low therapeutic index[3]. Hence, there is a major growing interest in current pharmacological research in Indian traditional system of medicine (natural origin) to develop new safest and efficient anticancer molecules to minimize the toxic effects of the existing medications[4,5]. Even the World Health Organization (WHO) and Food and Drug Administration (FDA) suggest the use of herbal medication as a complementary and alternative medicine for treatment of cancer and chemoprevention in human[6,7]. The several phytochemical agents like flavonoids, terpenoids, steroids etc have been taken as a great interest in drug design due to their multifunctional pharmacological activities including anticancer property[8]. The plant *Leucas indica* Linn. (Family: Labiatae) is commonly known as 'Dandokalos' in Bengali. It is distributed almost in every state of India but abundantly present in 'Mahananda Neora Valley' in West Bengal. It is an erect herb with pubescent branching. The leaves of this plant are linear-lanceolate in nature while the flowers are white with four stamens[9]. Traditionally, the leaves of this plant are used as vermifuge, stomachic, sedative and in sores[10]. The methanolic fraction of this plant showed significant wound healing activity[11]. The aqueous and methanolic extract of the aerial parts of *Leucas indica* having significant hypoglycemic, anti-inflammatory and antioxidant activity[12,13]. However, based on the literature survey and the lack of any research activities in anticancer field on this plant, the present study was designed to evaluate the anticancer potential by taking crude extracts of *Leucas indica* on EAC induced mice.

MATERIALS AND METHODS

Plant material

The aerial parts of *Leucas indica* Linn. were collected in August, 2011 from Duars region, Jalpaiguri District, West Bengal, India. It was identified and authenticated from Central National Herbarium, Botanical Survey of India, Howrah-711103, West Bengal (Vide No. CNH/32/2012/ Tech.II/625 Dated: 06.03.2012). After proper washing, it was dried under shade at a room temperature for seven days and then grinded with a mechanical grinder. Finally, the coarse powders were separated by sieving using 40 mesh apertures and stored in an air tight container for further use.

Preparation of plant extract

The fresh coarse powders were subjected to maceration by petroleum ether to remove fatty materials and then successively extracted with chloroform, ethyl acetate, methanol and distilled water according to ascending order of polarity of solvent using a Soxhlet apparatus. The each fraction of the extract was then filtered and concentrated to dryness in a rotary vacuum evaporator under reduced pressure and temperature and stored in desiccators. During performing the experiment, the dried methanloic and aqueous extract of *Leucas indica* were dissolved in distilled water to prepare the subsequent doses of extract (LIME and LIAE).

Chemicals and reagents

Trypan blue, methyl violet, sodium sulphate, methylene blue, sodium chloride, propylene glycol were purchased from MERCK Pvt. Ltd. (Mumbai, India) and Vinblastine Sulphate

NSHM Journal of Pharmacy and Healthcare Management
Vol. 03, February (2012) pp. 57-65

Hepatoprotective activity of ethanolic extract of *Aerva sanguinolenta* (Amaranthaceae) against paracetamol induced liver toxicity on Wistar Rats

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ABSTRACT The aim of this study is to investigate the Hepatoprotective effects of ethanolic extract of *Aerva sanguinolenta* (Family: Amaranthaceae) by oral route to adult male Wistar albino rats weighing 160-180gm.The protocol started with oral feeding of 200 and 400mg/kg body weight of extract and 25mg/kg body weight of Silymarin. After treatment of sixteen consecutive days and after 24-hours of last dose and 18-hours fasting, all animals in each group were sacrificed by cervical dislocation. The blood and liver were collected for biochemical estimation and histopathological observation. From the result it was found out that the ethanolic extract of the plant has hepatoprotective activity and that is comparable to that of Silymarin. Here hepatoprotective activity of ethanolic extract of *Aerva sanguinolenta* leaves may be due to the presence of polyphenolic compounds. Besides *Aerva sanguinolenta* contains flavonoid and tannin which are also known as natural antioxidants due to their electron donating property which either scavenge the principal propagating radicals or halt the radical chain.

Key words: *Aerva sanguinolenta*; silymarin; hepatoprotective; antioxidants

Introduction

The plant *Aerva sanguinolenta* (family-Amaranthaceae) is a perennial herb. The plant is available in tropical countries of Asia such as Bhutan, India, Nepal, Pakistan and also in China, Malaysia and Indo-China regions. In China it is known as Bai-hua-mi, in Maharashtra as Burval, in Uttarakhand as Sufedphulia and in Assam as Soru-araksan. In folklore medication leaf and flower of the plant were used as wound healing and anti inflammatory for injuries from falls, rheumatic arthritis and pain in muscles [1], the whole plant was used as diuretic and demulcent [2], tender shoot of the plant used as decoction form for galactogue to nursing mother [3] and decoction of whole plant was taken twice a day to expel intestinal worms [4].

Leaves and root of the plant have been used traditionally for body pain and the paste of leaf and root is applied to affected area [5].The plant extract showed significant wound healing property [6].

Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. So it has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [7].

Liver diseases are some of the fatal disease in the world today. They pose a serious challenge to international public health. Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for their treatment of liver disorders. But there is not much drug available for the treatment of liver disorders [8, 9].

The experimental intoxication induced by Paracetamol (640mg/kg body wt.) is

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Central European Journal of Experimental Biology, 2012, 1 (1):26-35
(http://scholarsresearchlibrary.com/archive.html)

ISSN: 2278–7364

Estimation of Biosurfactant Activity of an Alkaline Protease Producing Bacteria Isolated from Municipal Solid Waste

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ABSTRACT:

Biosurfactants are surface-active substances synthesized by microorganisms having the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable. Here an effort was made to screen biosurfactant activity of a protease producing bacteria isolated from municipal solid waste. Strain was identified as Pseudomonas aeruginosa by 16S rDNA based molecular technique. Biosurfactant, obtained from isolated organism was screened by hemolytic assay, drop collapsing method, oil spread method, blue agar plate method and oil spreading technique. In oil spread method kerosene oil shown the best result (58mm) and in emulsification index method petrol has given maximum efficient result (64 % in comparison to 1% of SLS having 58% EI). Besides biosurfactant activity the strain also produces protease enzyme. The strain has shown maximum protease activity at pH 9.5, temperature 37°C and 48 hrs. of incubation time. So, this strain can be used in textile, leather, detergent, pharmaceutical and dairy industries for its dual ability of producing protease enzyme and biosurfactant activity.

Key words: Biosurfactant, *Pseudomonas aeruginosa*, 16S rDNA based molecular technique.

INTRODUCTION

Recently, the products of biological origin, particularly enzymes, are attracting the attention of the researchers. Among the enzymes, proteases occupy an important position as they were the first to be produced in bulk, and now constitutes ~ 66% of total enzymes employed [1]. Proteases are present in all living organisms. But microbial proteases are most exploited group of industrial enzymes. Based on the mode of action, they are further classified into four categories viz. alkaline, acid, thiol and metallo proteases [2]. Since alkaline proteases are active over a broad pH (7-12) and temperature (35°C-80°C) ranges [3].

Protease is applied in pharmaceutical, food and detergent industries, waste treatment and others. Biotechnological importance of these enzymes has been realized by leather industries for the purpose of dehairing and bating hides as a substitute toxic chemical [4]. In food industry proteases are used as crude preparation. In pharmaceutical industry they are used as ingredients of ointments for debriment of wards and in medicine preparation [5].

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [6]. Unlike synthetic surfactants, microbially-produced compounds are easily biodegradable and suited for bioremediation and dispersion of oil spills. Potential application of biosurfactants includes emulsification, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic and pharmaceutical industries [7] and also possess antimicrobial activity against *Mycobacterium*

National Seminar on

AMRI HOSPITALS

"Clinical Pharmacology – Bench to Bedside"

Jadavpur University, Kolkata - 700 032, India

CERTIFICATE

This is to certify that

Mr./Ms./Dr./Prof. Mousumi Das

has attended as Delegate/Faculty in the National Seminar on **"Clinical Pharmacology – Bench to Bedside"**, held at Dr. Triguna Sen Auditorium, Jadavpur University, Kolkata on Friday 28th November, 2014 organized by Clinical Research Centre (CRC), Department of Pharmaceutical Technology, Jadavpur University in association with AMRI Hospitals, Kolkata.

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Faculty of Engineering and Technology, JADAVPUR UNIVERSITY

TEQIP-II SPONSORED ONE-DAY NATIONAL WORKSHOP

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This is to certify that Prof./ Dr./ Mr./ Mrs./ Ms. Mousumi Das
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Coordinator TEQIP-II	Nodal Officer, TEQIP-II R&D Committee	Workshop Coordinators

NOVARTIS

GCP Workshop

CERTIFICATE OF ATTENDANCE

This is to certify that

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has attended the GCP Workshop jointly conducted by Department of Hematology, Nil Ratan Sircar Medical College and Hospital and Novartis Oncology on 21st April 2017 at Kolkata, India

Dr Prantar Chakrabarti	*Dr Atul Chaudhari*	*Dr Manish Mistry*
Professor & Head, Dept. of Hematology, NRSMCH	Head, GMO India, Novartis Oncology	Medical Director, Novartis Oncology

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HAS PARTICIPATED IN

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