Systematic Exploration of Novel Heteroaryl Azole based Compounds and their Nanoparticles for Antiamoebic Therapeutics

Mohmmad Younus Wani

Centre for Interdisciplinary Research in Basic Sciences

Jamia Millia Islamia

New Delhi

December 2011

Systematic Exploration of Novel Heteroaryl Azole based Compounds and their Nanoparticles for Antiamoebic Therapeutics

Thesis
submitted to

JAMIA MILLIA ISLAMIA

In partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Chemistry**

by

Mohmmad Younus Wani

Under the supervision of

Dr. Fareeda Athar
Centre for Interdisciplinary
Research in Basic Sciences,
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CENTRE FOR INTERDISCIPLINARY RESEARCH IN BASIC SCIENCES
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December, 2011

Declaration

I, **Mohmmad Younus Wani**, student of Ph.D. hereby declare that the thesis titled "**Systematic Exploration of Novel Heteroaryl Azole based Compounds and their Nanoparticles for Antiamoebic Therapeutics**" which is submitted by me to the Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition. This is to declare further that I have also fulfilled the requirements of Para 8 (viii and ix) of the Ph.D. Ordinance, the details of which are enclosed at the end of the Thesis.

Place: New Delhi

Date: **Mohmmad Younus Wani**

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Abstract
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Broad Area of Research: Chemistry.

Special Field of Research: Organic/Medicinal Chemistry.

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Place: New Delhi

Date: **Mohmmad Younus Wani**

CERTIFICATE

On the basis of declaration submitted by Mohmmad Younus Wani, student of Ph.D., I hereby certify that the thesis titled "**Systematic Exploration of Novel Heteroaryl Azole based Compounds and their Nanoparticles for Antiamoebic Therapeutics**" which is submitted to the Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy, is an original contribution with existing knowledge and faithful record of research carried out by him under my guidance and supervision.

To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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Dr. Fareeda Athar
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Acknowledgement

(In the name of Allah, the beneficent, the merciful)

Read and thy Lord is the Most Bounteous ○ Who teach by the Pen ○ Teach man that which he knew not.

Hazrat Mohammad (Sullallah-u-Allahe-e-Wassalam) narrated

The most generous and munificent is Allah, then I am in the mankind and the person who learns and imparts knowledge, is the most generous after me.

*First of all I bow down my head to the omnipresent and omniscient **Almighty Allah**; as a result of His clemency my success was finally consummated.*

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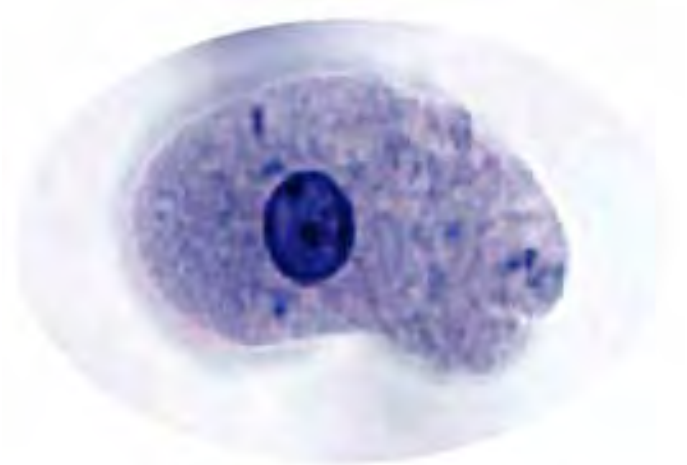
Mohmmad Younus Wani

Dedicated to My Parents

Contents

Chapter 1

General Introduction



Entamoeba histolytica

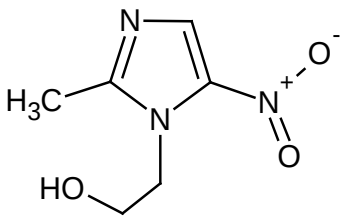
1.1. INTRODUCTION

Undoubtedly, tropical diseases today are a major health problem. They affect more than two billion people worldwide (about one third of the total population) and cause nearly two million deaths per year, mostly in the poorest areas of the planet [1, 2]. The wide diffusion of these diseases, the small number of effective drugs and the frequent emergence of resistance make drug discovery in this therapeutic area a very urgent and challenging task. Among the several known tropical diseases, Amoebiasis, caused by protozoan parasite *Entamoeba histolytica* [3] is the third leading parasitic cause of death worldwide, surpassed only by malaria and schistosomiasis [4, 5]. Occasionally, *E. histolytica* trophozoites penetrate the intestinal mucosa, causing amoebic colitis and spread via portal circulation to other organs, most commonly to liver, where they induce amoebic liver abscess (ALA), the most common extra intestinal manifestation of invasive amoebiasis [6]. Majority of amoebic liver abscess patients have a single abscess of variable sizes located in the right lobe, predominantly within segment six to eight [7]. In most cases, amoebic liver abscess can be efficiently treated by intake of metronidazole alone [8], but some recent studies have shown that this drug have several toxic effects such as genotoxicity, gastric mucus irritation, and spermatozoid damage [9, 10]. Furthermore, failures in the treatment of several intestinal protozoan parasites may result from drug resistant to parasites [11, 12].

Azoles, five-membered heterocyclic compounds with two or three nitrogen atoms, constitute a large group of organic substances exhibiting a wide range of biological activities. For example, imidazole is a part of the molecules of histidine, an important amino acid, and histamine, the product of its decarboxylation. Imidazole derivatives possess potential biological activities such as antihelmintic, antiamoebic, antiparasitic,

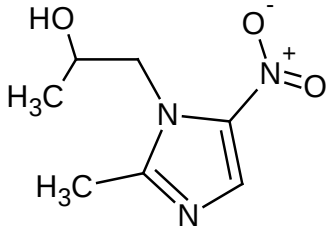
antiprotozoal, anti-inflammatory, analgesic, antifungal and antibacterial [13-15]. The introduction of azole based drugs in the late 1950's and 1960's heralded a new era in the treatment of gram negative and gram positive bacteria and a range of pathogenic protozoan parasites. Azomycin (a 2-nitroimidazole), an antibiotic isolated in Japan from a streptomycete, was the first active compound of the nitroimidazoles to be discovered [16] and acted as the main impetus for the systematic search for drugs with activity against anaerobic protozoa. This led to the synthesis of the 5-nitroimidazole, metronidazole *(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol)* and the demonstration of activity against Trichomonas by [17]. Subsequently, metronidazole was demonstrated to cure giardiasis [18], amoebiasis [19] and Balantidium infections [20]. The first indication of activity of metronidazole against bacteria came from studies on a patient with acute ulcerative gingivitis who was treated for concurrent trichomoniasis with metronidazole. The 5-nitroimidazoles are now widely used as a prophylaxis against post-operative sepsis and in the treatment of gastric ulcers caused by Helicobacter pylori [21]. Metronidazole is the most extensively used 5-nitroimidazole and is currently the recommended treatment for protozoan infections of *Giardia intestinaIis* [22], *Trichomonas vaginalis* [23], *Entamoeba histolytica* [24] *Blastocystis hominis* [25] and has also been used to treat Leishmania [26]. As an antibacterial agent it has been successfully used to treat *Helicobacter pylori*, Bacteroides spp., Eubacterium spp., Peptococcus spp., Peptostreptococcus spp., Clostridium spp., Fusobacterium spp. and *Gardnerella vaginalis* [27, 28]. Many other nitroimidazoles such as tinidazole [29], nimorazole [30], ornidazole [31, 32], satranidazole [33], secnidazole [34], nimorazole and carnidazole [35] have been used clinically or experimentally to treat the anaerobic, pathogenic protozoa and bacteria in human and veterinary medicine **(1.1)**. Metronidazole was first introduced into clinical

medicine in **1959** for the treatment of *Trichomonas vaginalis* infections [2, 36] and it has been subsequently used for invasive amoebiasis since 1966 [19] and has since then remained the gold standard for amoebiasis treatment. Metronidazole needs to be reduced in the parasite in order to become active [37]. In trichomonads, reduced ferredoxin formed in the pyruvate: ferredoxin oxidoreductase (PFOR) reaction is believed to reduce metronidazole. *In vitro* data corroborate this hypothesis [38]. Since PFOR is also an abundant enzyme in *E. histolytica*, the same mechanism is suggested to exist in *E. histolytica*. It has also been reported that the flavin enzyme thioredoxin reductase is also able to reduce and activate metronidazole and other nitro compounds [39]. The activated reaction products, such as the nitroradical anion and further reduced intermediates then attack cellular components of the parasite in particular proteins and other thiol containing compounds.



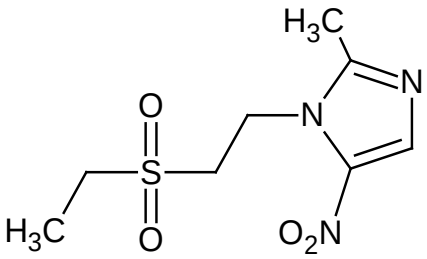
2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol

Metronidazole



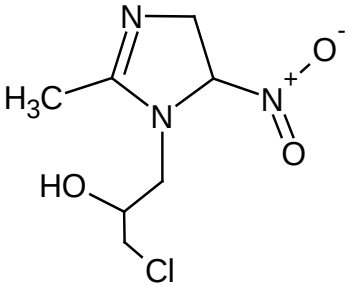
1-(2-methyl-5-nitro-1*H*-imidazol-1-yl)propan-2-ol

Secnidazole



1-[2-(ethylsulfonyl)ethyl]-2-methyl-5-nitro-1*H*-imidazole

Tinidazole

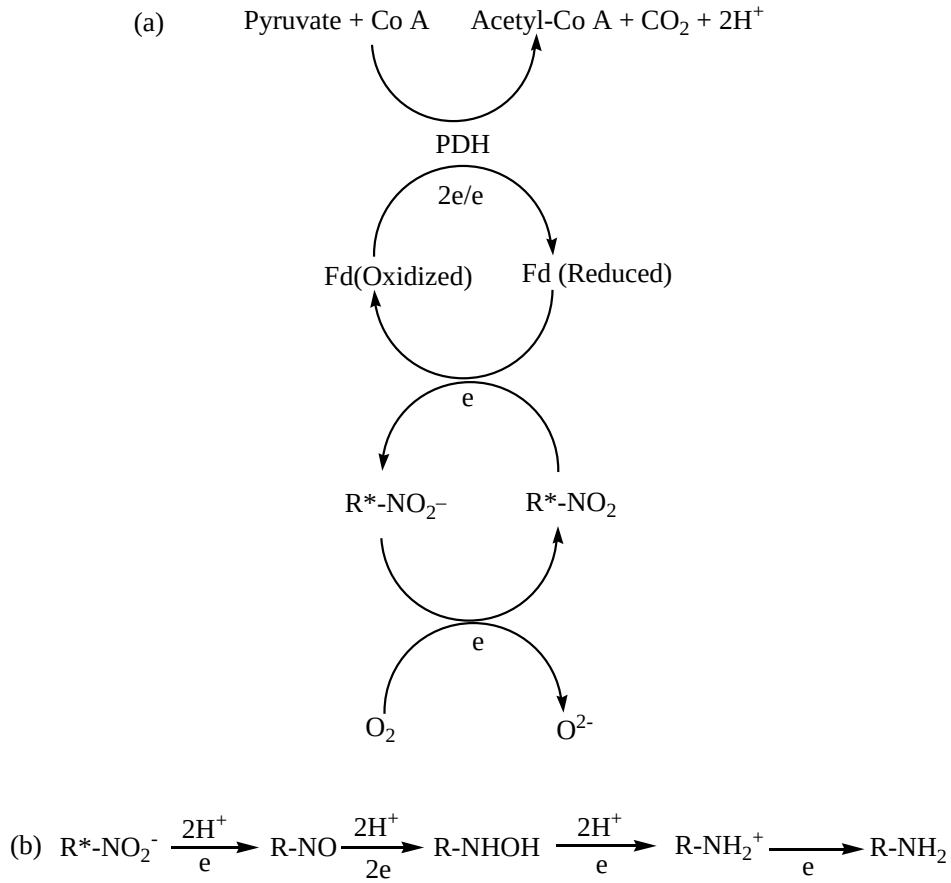


1-chloro-3-(2-methyl-5-nitro-4,5-dihydro-1*H*-imidazol-1-yl)propan-2-ol

Ornidazole

Tissue Amoebicides

Azole based antiamoebic drugs **(1.1)**.



Proposed pathway of metronidazole reduction in anaerobic protozoa including Entamoeba histolytica **(1.2)**.

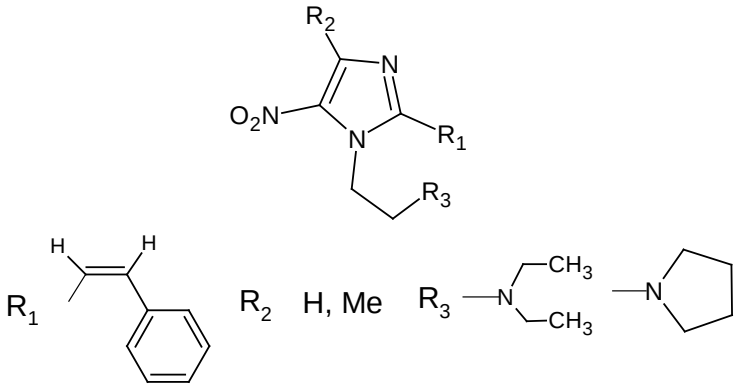
(**a**) Ferredoxin is reduced by pyruvate:ferredoxin oxidoreductase (PFOR) which accepts electrons from pyruvate. Metronidazole is subsequently reduced in a one-electron step to the unstable oxygen reactive intermediate (*R-NO_2) by reduced ferredoxin. Oxygen may in turn oxidise reduced metronidazole leading to futile cycling of the drug and the formation of superoxide. Hydrogenase may replace PFOR in the reduction of ferredoxin. Ferredoxins may be reduced by one or two electrons (2e/e) depending on the number of iron-sulphur clusters in the protein. Flavodoxin can serve as an electron acceptor from hydrogenase or PFOR and replace ferredoxin in the reduction of metronidazole. (**b**) A two-electron reduction of Metronidazole

leads to the formation of the highly toxic nitroso intermediate (R-NO). Further reduction results in the hydroxylamine radical ion and finally the amine.

1.2. Azole based antiamoebic compounds

1.2.1. Imidazoles

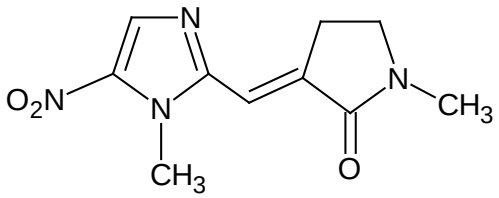
Imidazole is a planer five-member heterocyclic ring and a constituent of several important natural products, including purine, histamine, histidine and nucleic acid. Being a polar and ionisable aromatic compound, it improves pharmacokinetic characteristics of lead molecules and thus used as a remedy to optimize solubility and bioavailability parameters of proposed poorly soluble lead molecules. A series of N-alkylaminonitroimidazoles was synthesized by Giraldi et al., [40, 41] by refluxing the sodium salt of nitroimidazoles with N-substituted aminoethyl chlorides in 35-89% yield. Two possible isomeric products 1-aminoalkyl-5(4)-nitroimidazoles were obtained and examined for their antiamoebic activity. All the compounds showed very interesting biological activity, and the introduction of a styryl group in one of the compounds **(1.3)** showed enhancement of the antiamoebic activity of these nitroimidazoles.



(1.3)

A series of 5-nitroimidazoles was prepared by Upcroft et al., using literature procedures and tested them against 3 protozoan parasites including *E. histolytica* [42]. One of the compounds, 1-methyl-3-(1-methyl-5-nitro-1H-imidazol-2-ylmethylene)

pyrrolidin-2-one, was found to be active (minimum lethal concentration >5 μM) against *E. histolytica* **(1.4)**.

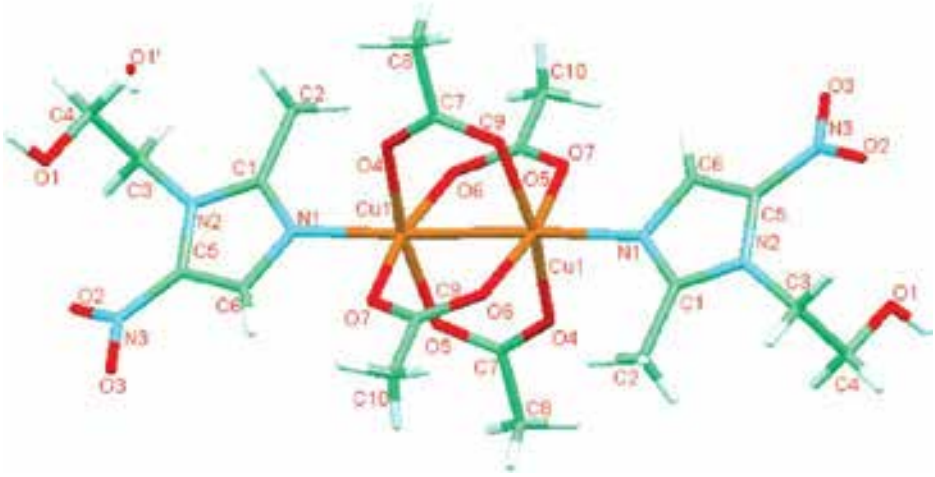


(3*E*)-1-methyl-3-[(1-methyl-5-nitro-1*H*-imidazol-2-yl)methylidene]pyrrolidin-2-one

(1.4)

Complexation of Metronodazole, a 5-nitroimidazole with different transition metals has also been studied by different workers. Bharti and others have synthesized and screened a series of series of Pd, Pt, Cu, Au, and Ru complexes of metronidazole against *E. histolytica* [43, 44]. The results of this study show that the ratio of IC_{50} of all complexes to metronidazole were 4-18-fold, which indicated that the complexes were far more active than metronidazole. These studies have further shown that inhibition was mostly due to the presence of metal complexed metronidazole. The palladium **(1.5)** and copper metronidazole **(1.6)** complexes demonstrated higher activity which proved the fact that metal incorporation enhances the activity of the drug both *in vitro* and *in vivo*.

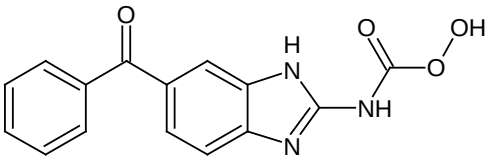
$[PdCl_2(mnz)_2]$ (1.5)



$[Cu_2(OAc)_4(mnz)_2]$ (1.6)

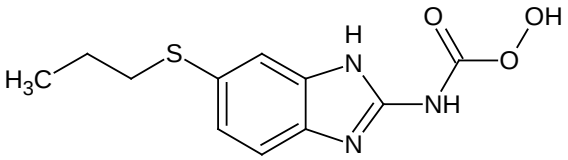
1.2.2. Benzimidazoles

Benzimidazole is a heterocyclic aromatic organic compound. This bicyclic compound consists of the fusion of benzene and imidazole. The most prominent benzimidazole compound in nature is *N-ribosyl-dimethylbenzimidazole*, which serves as an axial ligand for cobalt in vitamin B_{12}. Benzimidazole derivatives, such as mebendazole and albendazole, are clinically anthelminthic useful drugs **(1.7)**. Recently, antiprotozoal activity of 2- and 5-substituted benzimidazoles has been reported [45, 46].



5-benzoyl-2-(carboperoxyamino)-2,3-dihydro-1*H*-benzimidazole

Mebendazole



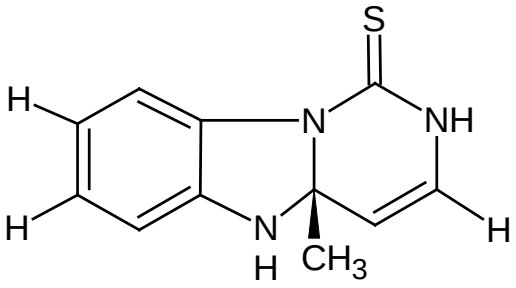
2-(carboperoxyamino)-6-(propylsulfanyl)-1*H*-benzimidazole

Albendazole

(1.7)

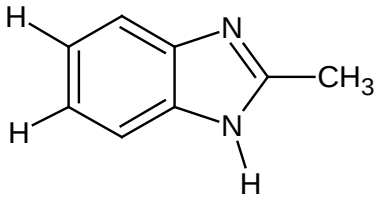
Benzimidazole core is of a wide interest and is a well-known privileged structure in medicinal chemistry because of its diverse biological activities and is widely used in searches for new drugs [47, 48].

Sondhi et al., described one-step synthesis of pyrimido benzimidazoles, in which isothiocyanatobutanal was condensed with o-phenylenediamine in refluxing methanol at pH≈ 5 to give pyrimidobenzimidazole derivatives in 18-46% yield [49]. In this series, most of the compounds showed good biological activity and one of the compounds **(1.8)** had IC_{50} value comparable to metronidazole (IC_{50}= 1.45µM).



(1.8)

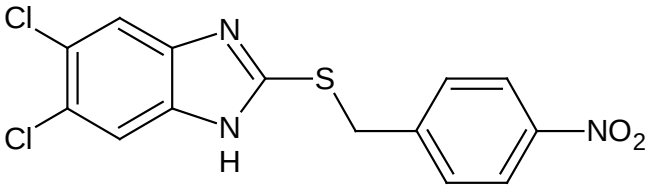
Valdez et al., synthesized a series of 25 benzimidazole derivatives from substituted 1,2-phenylamine intermediates [50]. Biological assay results against *E. histolytica,* indicated that, with very few exceptions, most of the benzimidazole derivatives demonstrated higher activity than metronidazole. One compound **(1.9)** was obtained with 70 times more antiamoebic potency than metronidazole.



(1.9)

Kazimierczuk et al., [51], prepared two series of nitro and halogen-substituted benzimidazole derivatives by reaction of various substituted benzimidazoles with appropriate halogenoalkylamines in acetonitrile using 1,8 diazobicyclo[5,4,0]undec-7-

en as a base. Antibacterial and antiprotozoal activity of the newly obtained compounds were studied, and out of 10 compounds, only 5,6-dichloro-2-(4-nitrobenzylthio)benzimidazole **(1.10)** was found to be active against *E. histolytica*.



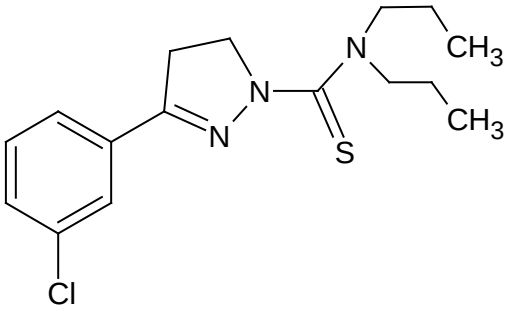
5,6-dichloro-2-[(4-nitrobenzyl)sulfanyl]-1*H*-benzimidazole

(1.10)

1.2.3. Pyrazole/Pyrazolines

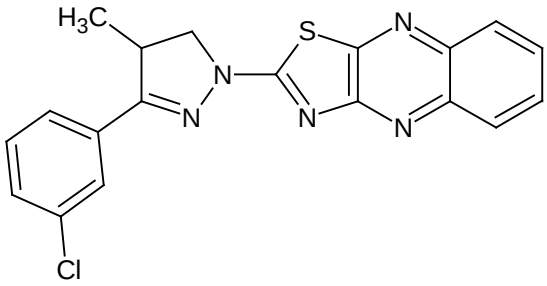
Pyrazole refers both to the class of simple aromatic ring organic compounds of the heterocyclic diazole series characterized by a 5-membered ring structure composed of three carbon atoms and two nitrogen atoms in adjacent positions, and to the unsubstituted parent compound. Pyrazolines have been reported as human acyl CoA cholesterol acyltransferase [52] as well as low-density lipoprotein oxidation inhibitors [53]. Moreover, 1,3,5-triaryl-2-pyrazolines were reported to possess antidepressant properties [54] in addition to monoamine oxidase inhibitory activities [55].

Abid et al., have synthesized series of 1-N-substituted pyrazoline analogues by the cyclization of Mannich bases with substituted thiosemicarbazides in 9-28% yield [56]. Screening of the resulting pyrazoline derivatives against *E. histolytica* revealed that all the 3-bromo- and 3-chlorophenyl substituted cyclized pyrazolines were more active than their respective unsubstituted analogues. Among the series one compound **(1.11)** was obtained as highly antiamoebic as compared to metronidazole.



(1.11)

In another similar study Abid et al., have synthesized some1-(thiazolo[4,5-b]quinoxaline-2-yl)-3-phenyl-2-pyrazoline derivatives and screened them for their antiamoebic activity [57]. The pyrazoline derivatives showed an IC_{50} value in the range 17.2–4.4 l µM. The conversion of these compounds into quinoxaline derivatives resulted in the increase of their antiamoebic activity. They showed IC_{50} in the range 6.76–0.72 µM. Compound bearing 3-chlorophenyl and 4-methyl substitutions on the pyrazoline ring (**1.12**) was the most active among the series.

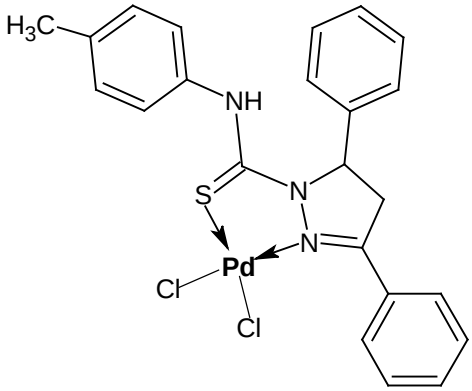


2-[3-(3-chlorophenyl)-4-methyl-4,5-dihydro-1*H*
-pyrazol-1-yl][1,3]thiazolo[4,5-*b*]quinoxaline

(1.12)

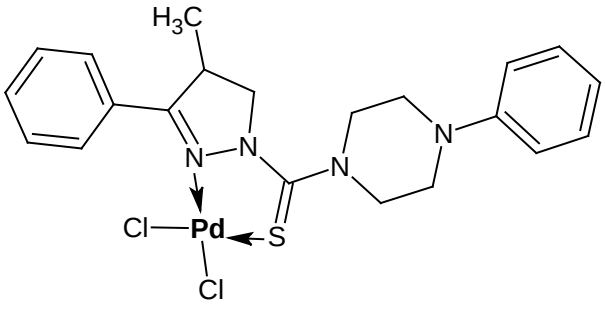
Budakoti et al., [58] reported, a series of 1-N-substituted thiocarbamoyl-3,5-diphenyl-2-pyrazoline derivatives against *E. histolytica*. First, a base-catalyzed Claisen-Schmidt condensation of benzaldehyde with acetophenone produced the chalcone, which on cyclization with various N-4 substituted thiosemi-carbazides gave the desired pyrazoline derivatives. The palladium (II) complexes were also prepared by mixing an equimolar ratio of the pyrazoline with $[Pd(DMSO)_2Cl_2]$ in 76-92% yield.

The IC_{50} values of the pyrazolines were found to be in the range of 0.38-11.02 μM. One of the Pd(II) complexes **(1.13)** has shown better IC_{50} as compared to the reference drug.



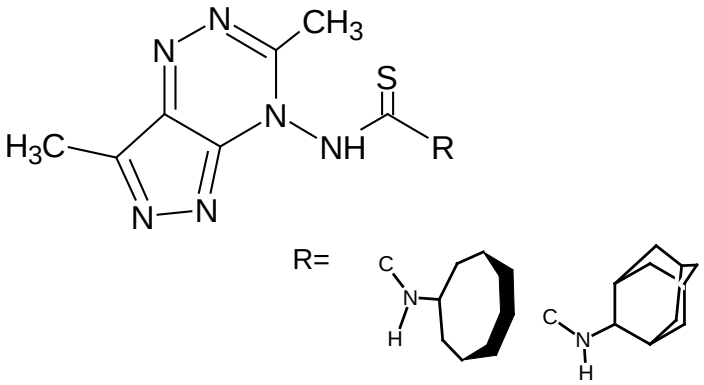
(1.13)

Kakul et al., have also reported the synthesis and antiamoebic activity of some pyrazoline derivatives [59]. These Pyrazoline derivatives have been later converted into palladium(II) complexes and studied for their antiamoebic behavior. Results obtained revealed that the presence of bulky groups at position N^4 of the thiosemicarbazone moiety greatly enhanced the antiamoebic activity, which was further enhanced due to the complexation of cyclised pyrazoline thiosemicarbazones with palladium(II). (IC_{50} = 0.37-5.65 μM as compared to reference drug Metronidazole (IC_{50} = 1.80 μM)). The most active compound in this series has been found to be **(1.14)** with IC_{50} value 0.37 μM.



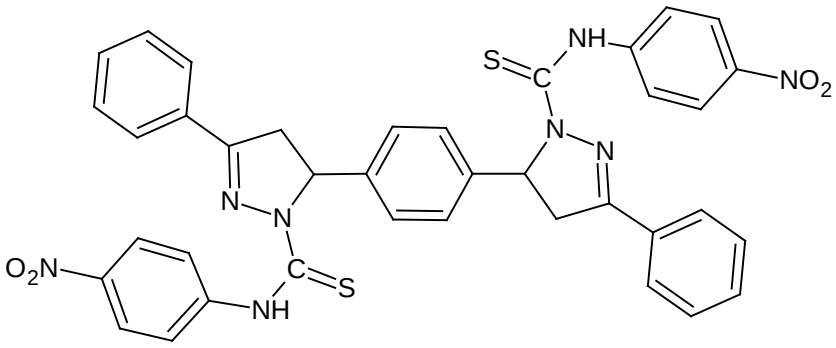
(1.14)

Shailendra et al., have synthesized a series of 3,7-dimethyl-pyrazolo[3,4-e][1,2,4]triazin-4-yl thiosemicarbazide derivatives and evaluated them *in vitro* against *HM1:1MSS* strain of *Entamoeba histolytica* [60]. The synthesized compounds exhibited antiamoebic activity in the range (IC_{50} = 0.81-7.31 µM). It has been inferred from the *in vitro* studies that some compounds **(1.15)** were found to be significantly better inhibitors of *E. histolytica* since IC_{50} values in the µM range elicited by these compounds are much lower than metronidazole.



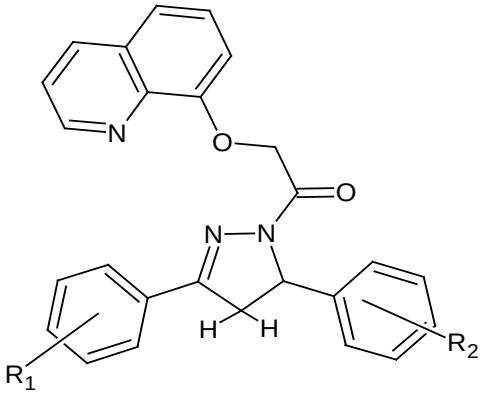
(1.15)

Bhat et al., have reported the synthesis of Bis-pyrazolines as *Entamoeba histolytica* growth inhibitors [61]. These Bis-pyrazolines have been synthesized by the cyclization of chalcone with N-4 substituted thiosemicarbazides under basic conditions. One compound **(1.16)** having IC_{50} = 0.42µM showed the most promising results out of all the compounds screened.



(1.16)

In a recent study F. Hayat et al., synthesized a series of Pyazolines bearing a quinoline tail and screened them for their antiamoebic activities [62]. The cyclization of different chalcones with 2-(quinolin-8-yloxy) acetohydrazide under basic condition led to the formation of new compounds; pyrazoline derivatives. The pyrazoline derivatives showed IC_{50} values in the range 0.05-16.3 µM. Among all the compounds two compounds **(1.17)** showed excellent antiamoebic activity (IC_{50} = 0.05-0.06 µM)



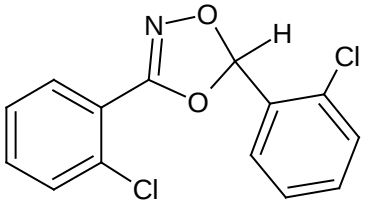
R_1 4-Cl, H

R_2 4-CH_3, 4-OCH_3

(1.17)

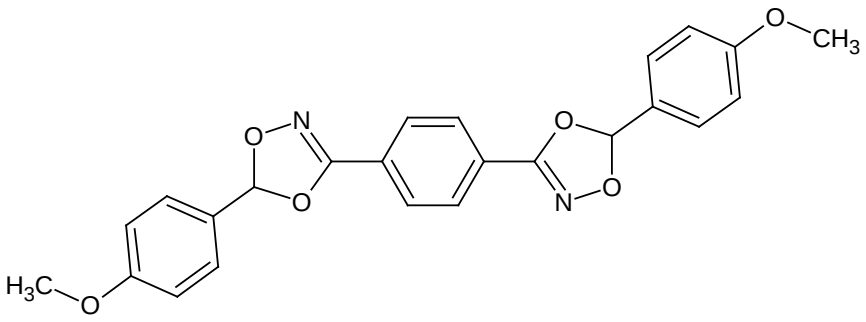
1.2.4. Dioxazoles

Dioxazole, an important member of the azole family also contains a number of biologically active molecules, which play an important role in the drug chemistry. Bhat et. al report some new derivatives of 3,5-substituted-1,4,2-dioxazoles with antiamoebic activity [63]. Among the series, some compounds, including compound **(1.18)** showed better antiamoebic activity than metronidazole with IC_{50} values in the range 0.41-1.73 µM and 1.80 µM respectively.



(1.18)

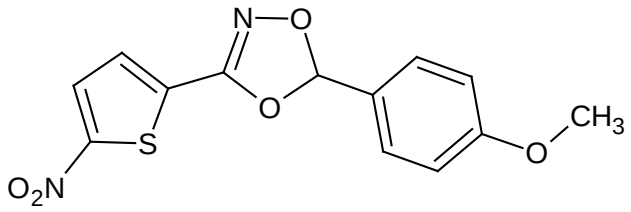
In another similar study Iqbal et al., reported synthesis of some Bis-dioxazole derivatives, obtained through the cyclization of benzene-1,4-dicarbaldehyde dioxime with different aromatic aldehydes in inert atmosphere as potential antiamoebic agents [64]. Compounds shoed activity in the range of (IC_{50}= 1.01-1.41 µM). The most active compound **(1.19)** in this series which has shown promising results with IC_{50} value of 1.01 µM has methoxy group at para position of the phenyl ring.



5-(4-Methoxyphenyl)-3-(4-(5-(4-methoxyphenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole

(1.19)

Recently Irfan et. al., reported some new dioxazole derivatives as *Entamoeba histolytica* and *Giardia intestinalis* inhibitors [65]. It is interesting to note that the compounds found active against *E. histolytica* were not found active against *G. intestinalis.* Compounds showed activity in the range of 1.60–9.97 µM and one compound (**1.20)** was obtained as highly antiamoebic (IC_{50} = 1.60 µM).



5-(4-methoxyphenyl)-3-(5-nitrothiophen-2-yl)-1,4,2-dioxazole

(1.20)

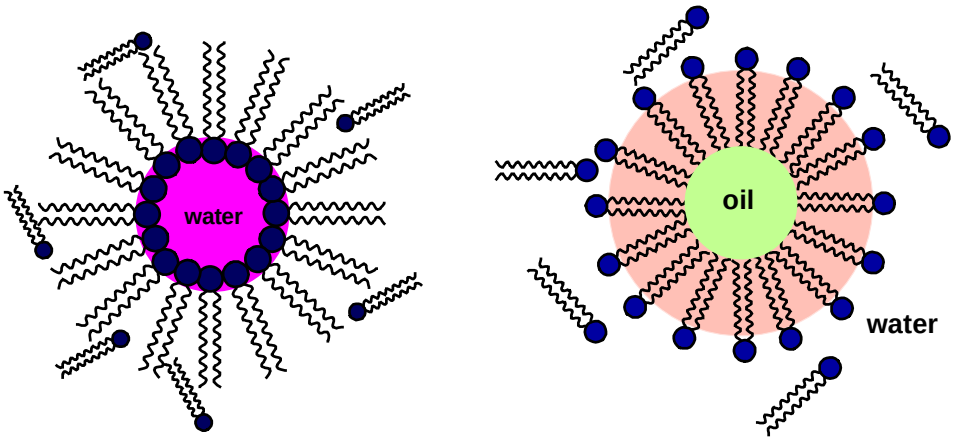
1.3. Synthesis of Nanoparticles

The research in nanoengineering of hydrophobic drugs has received great attention in the last few years due to their ability to satisfy the regulatory requirement with much less efforts as compared to carrier-based nanoparticulate systems and also due their various potential applications. Researchers are currently engaged in employing nanoparticles to deliver drugs, to seek out cancer cells before tumors grow, remove and/ or replace "broken" parts of cells or cell mechanisms with miniature, molecular-sized biological "machines", and use similar "machines" as pumps or robots to deliver medicines when and where needed within the body. The nanosize of these systems allows crossing of biological barriers, ameliorate tissue tolerance and improves cellular uptake and transport, thus enabling efficient delivery of the therapeutic agents to the target sites like liver, brain and solid tumor. Additionally, the nanoparticulate systems have revealed a great potential in tackling problems like low oral bioavailability, high intersubject variability and poor or suboptimal therapeutic response associated with delivery of poorly water-soluble drugs. The submicronic nature of these systems helps in improving therapeutic and pharmacokinetic performance of such drugs, thus enabling effective drug delivery by various routes, especially parenteral and peroral.

A variety of physical and chemical methods like chemical reduction methods [66-69] thermal methods [70] irradiation methods [71] or methods using laser ablation [72] have been employed for the synthesis of nanoparticles. Among these methods microemulsion method is one of the versatile preparation technique which enables control of particle properties such as size, geometry, morphology, homogeneity and surface area [73, 74].

1.3.1. Microemulsion Method

Microemulsions are isotropic, macroscopically homogeneous, and thermodynamically stable solutions containing at least three components, namely a polar phase (usually water), a nonpolar phase (usually oil), and a surfactant. A "water-in-oil" micro emulsion **(1.21)** is formed when water is dispersed in a hydrocarbon based continuous phase. Micelles in these systems can be described as "nanoreactors", providing a suitable environment for controlled nucleation and growth **(1.21)**. In addition, at the latter stages of growth, steric stabilization provided by the surfactant layer prevents the nanoparticles from aggregating [75]. Normal micelles can solubilize more oil in the hydrocarbon core, forming swollen micelles which are oil-in-water (o/w) microemulsions **(1.21)**.

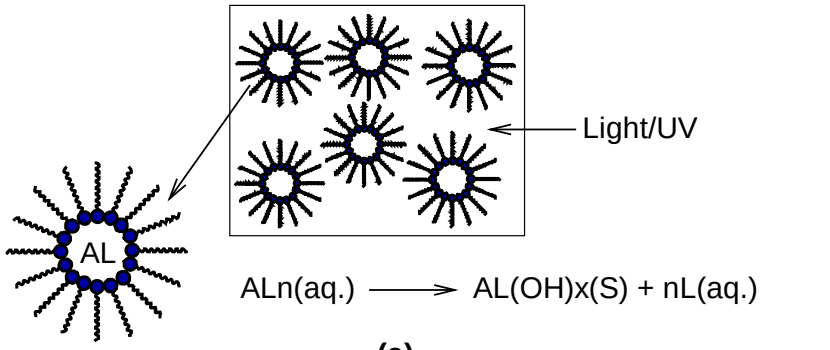


Water in Oil microemulsion **Oil in water microemulsion**

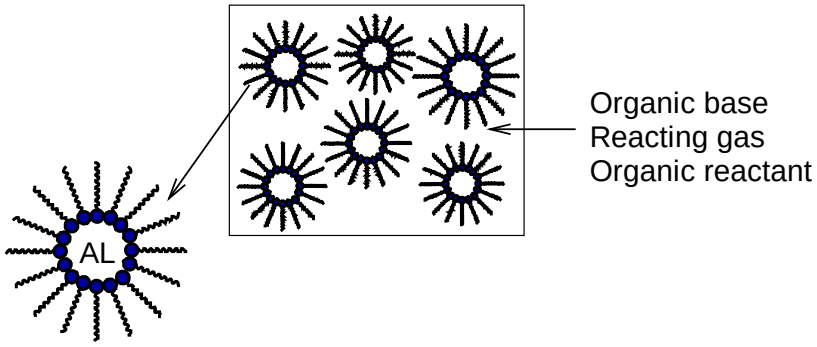
(1.21)

In general there are two methods for nanoparticle synthesis using microemulsion techniques [76]. The first method is called the one microemulsion method. This method includes "energy triggering" and the "one micro emulsion plus reactant" method. In the energy triggering method, the reaction is initiated by implementing a triggering agent into the single micro emulsion which contains a reactant precursor **(1.22a)**. However in one microemulsion plus reactant method, the reaction is initiated

by directly adding the pure reactant (liquid or gaseous phase) into the microemulsion containing another reactant **(1.22b)**. The one-micro emulsion method generally is driven by the diffusion-based process, since the second trigger/reactant is diffusing into the droplets containing the reactant in the used microemulsion.



(a)



$AM(aq.) + N(OH)\ (org.) \longrightarrow A(OH)x\ (s) + M^-\ (aq.) + N(aq.)$

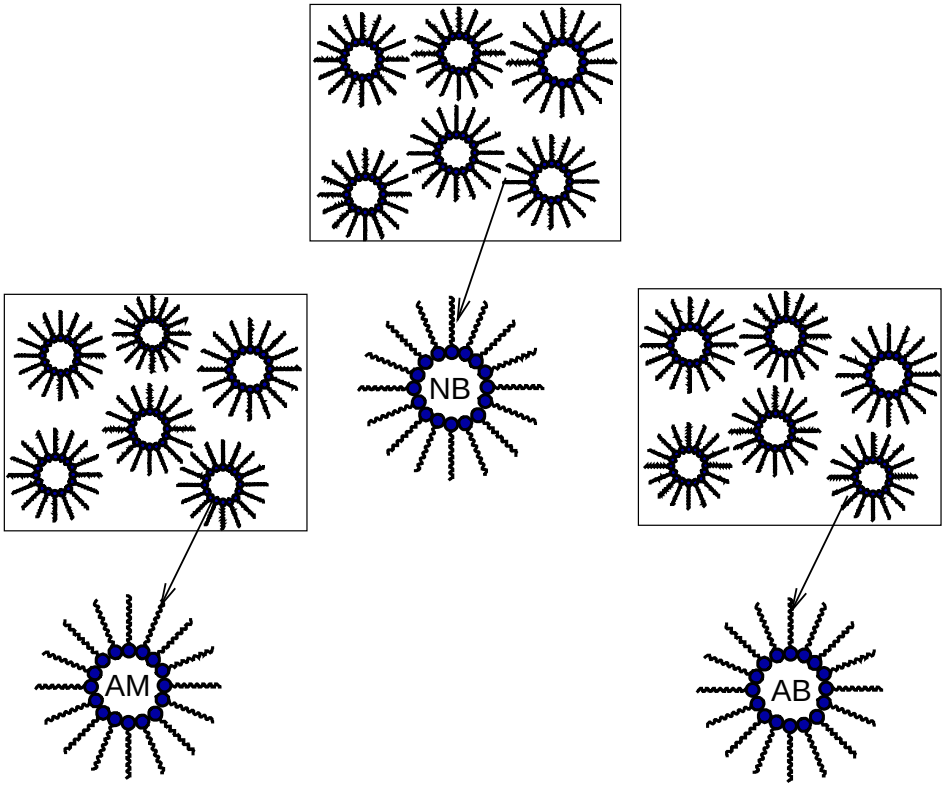
$AM(aq.) + B\ (g) \longrightarrow AB(s) + M^-\ (aq.)$

$x\ H_2O(aq.) + A(RO)x\ (org.) \longrightarrow AO_{x/2} + xROH$

(b)

Different methods of nanoparticle synthesis in micro emulsions: ***1.22a.*** *One microemulsion method: Energy triggering method.* ***1.22b.*** *One-micro emulsion method plus reactant method.*

The second method which is often used for preparing nanoparticles is the two micro emulsion methods. Two reactants A and B which are dissolved in the aqueous nanodroplets of two separate micro emulsions are mixed as shown in **(1.23)**. This method relies on fusion-fission events between the nanodroplets.

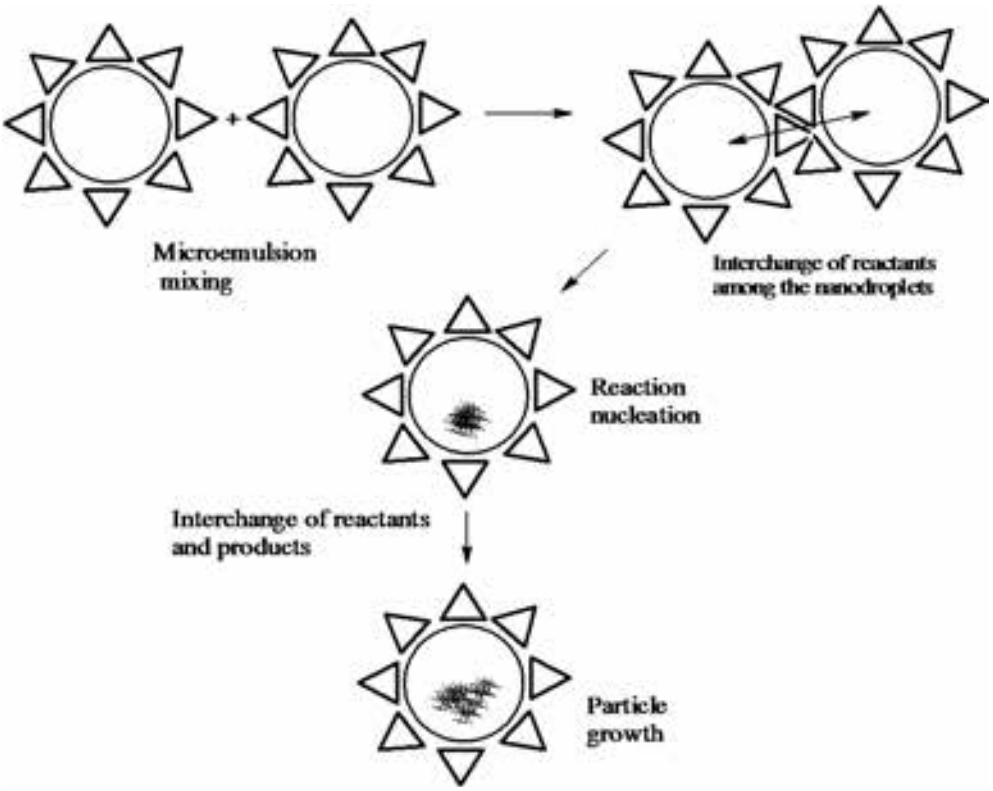


$$AM(aq.) + NB(aq.) \longrightarrow AB(s) + MN(aq.)$$

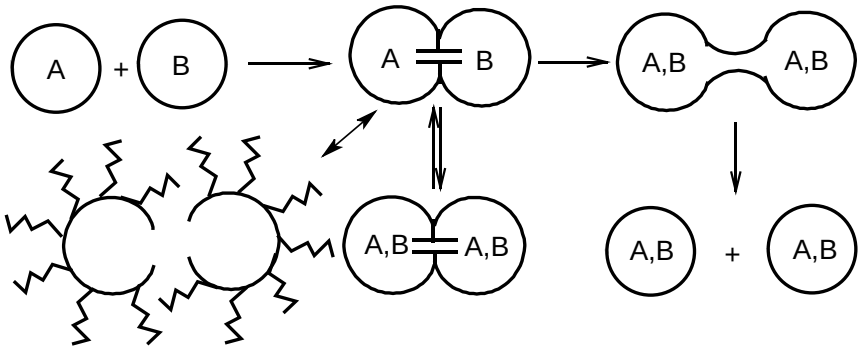
Two-microemulsion method. Cations: A, N, Anions: B, M **(1.23)**.

1.5.2. Mechanism of nanoparticle synthesis

The aqueous droplets continuously collide, coalesce, and break apart, resulting in a continuous exchange of solution content. In fact, the half-life of the exchange reaction between the droplets is of the order of 10^{-3}-10^{-2}s [77]. The different stages of nanoparticle formation process inside water droplets can be explained as: chemical reaction, nucleation and particle growth. Figure **(1.24)** and **(1.25)** explains the mechanistic steps involved in the formation of nanoparticles.



Mechanism of the synthesis of nanoparticles in microemulsions **(1.24)**



Mechanism for the formation of equal number of droplets **(1.25)**

Taking inspiration from the above mentioned facts this research work aimed to synthesize some new libraries of heterocyclic compounds and evaluate them for their antiamoebic properties, because there is an urgent need for the synthesis of some better antiamoebic drugs. Efforts have also been made in the nanosizing of some of these compounds to study the effect on the biological behavior of these molecules under study.

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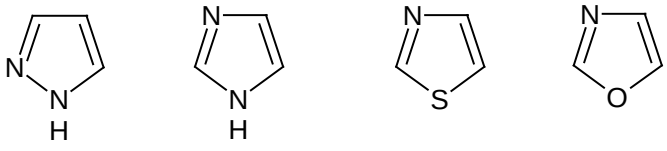
Chapter 2

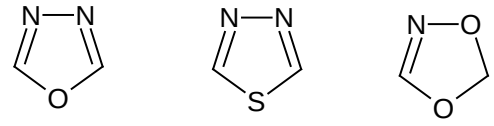
Synthesis, Characterization and Antiamoebic activity of terpene based 1,4,2-Dioxazole derivatives

2.1. INTRODUCTION

An azole is a class of five-membered nitrogen heterocyclic ring compounds containing at least one other non-carbon atom of either nitrogen, sulfur, or oxygen [1]. The parent compounds are aromatic and have two double bonds; there are successively reduced analogs (azolines and azolidines) with fewer. One, and only one, lone pair of electrons from each heteroatom in the ring is part of the aromatic bonding in an azole. Names of azoles maintain the prefix upon reduction (e.g., pyrazoline, pyrazolidine). Some of the common azoles include Pyrazole **(2.1)**, Imidazole **(2.2)**, thiazole **(2.3)**, oxazole **(2.4)**, Oxadiazole **(2.5)**, thiadiazole **(2.6)**, dioxazole **(2.7)**. The numbering of ring atoms in azoles starts with the heteroatom that is not part of a double bond, and then proceeds towards the other heteroatom.

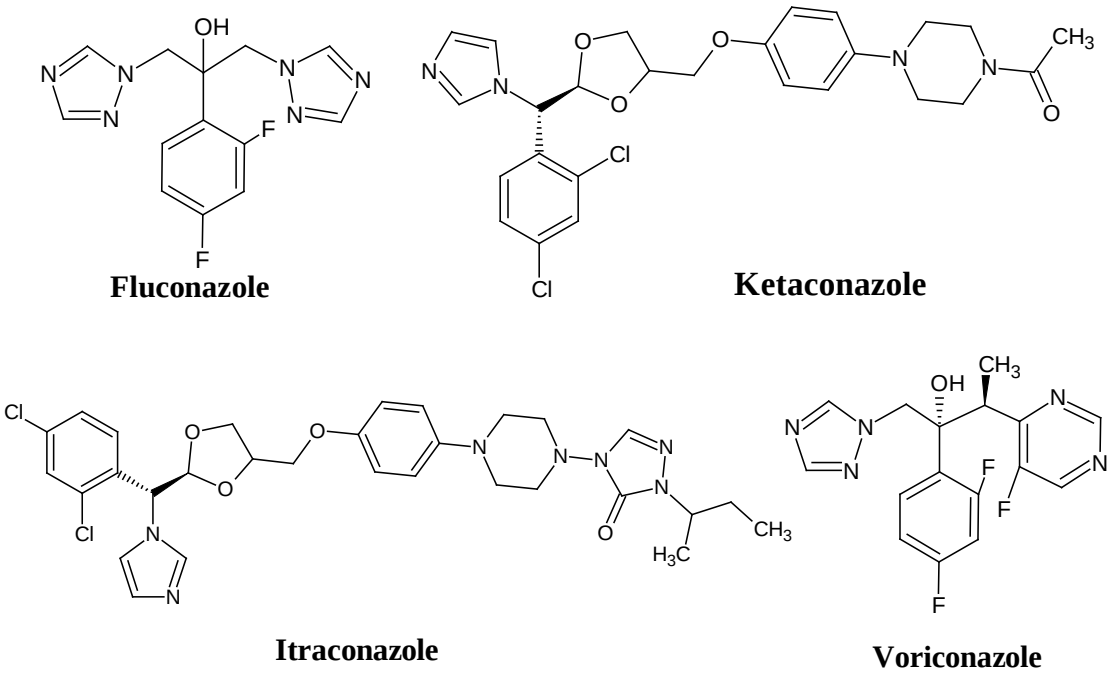


(2.1) **(2.2)** **(2.3)** **(2.4)**



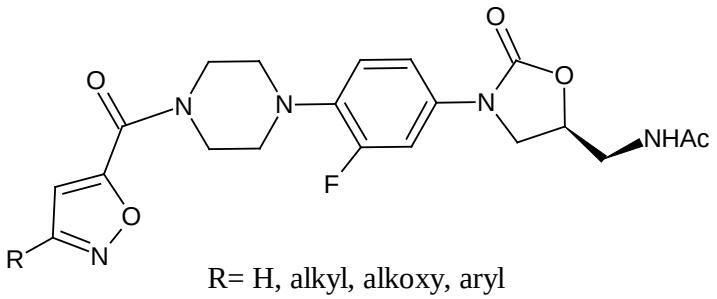
(2.5) **(2.6)** **(2.7)**

Among the heterocycles, the nitrogen-containing five-membered heterocycles are especially important since they have a variety of bioactivities. Additionally, aromatic nitrogen heterocycles with oxygen as in isoxazoles, oxazoles, 1,3,4-oxadiazoles, and 1,2,4-oxadiazoles are also of great importance in drug synthesis. Most of the commonly antimicrobial drugs currently in use are Fluconazole, Ketoconazole, itraconazole, Voriconazole **(2.8)** contain this ring in their skeleton.



Commonly used Azole based antimicrobial drugs **(2.8)**.

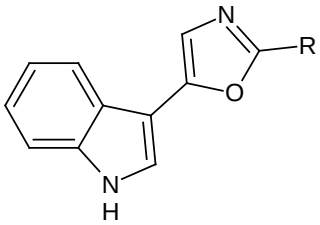
Oxazole an important member of the azole family contains a number of biologically active molecules, which play an important role in drug chemistry. Classically, the Robinson-Gabriel synthesis was the most common route to oxazoles, which involves dehydration of 2-acylamino-ketones [2]. A versatile extension of this cyclodehydration reaction was developed by Wipf and Miller [3] which involved either an oxidation followed by cyclodehydration or cyclodehydration of hydroxy amides with subsequent oxidation under mild conditions [4]. Two series of oxazolidinone derivatives **(2.9)**, having substituted isoxazoles were synthesized and tested for antibacterial activities against several Gram-positive strains including the resistant strains of *Staphylococcus* and *Enterococcus*. Some of them showed *in vitro* activities (MIC) comparable or superior to the reference compound vancomycin [5].



R= H, alkyl, alkoxy, aryl

(2.9)

Many natural products containing 5-(3′-indolyl)oxazole ring system such as pimprinine **(2.10)**, pimprinethine **(2.11)**, pimprinaphine **(2.12)** have been isolated and identified [6-9]. Pettit and co-workers have reported Laboradorin 1 **(2.13)** and Laboradorin 2 **(2.14)**, isolated from *Pseudomonas syringae* pv. Coronafaciens as growth inhibitors of various cancer cell lines [10]. The isomeric 4-(3′-indolyl)oxazole represent a common structural moiety of many biological potent natural products such as phenoxan [11], calyculins [12] and rhizoxin [13]. Recently a group of workers reported synthesis of some indolyl-1,3,4-oxadiazoles **(2.15)** and discovered that some of the analogs are potent and selective against various cancer cell lines [14].



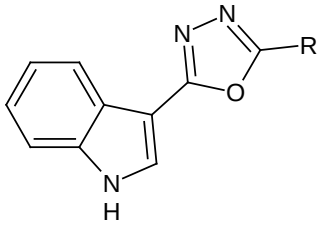
2.10 Pimprinine R= CH_3

2.11 Pimprinethine R= CH_2CH_3

2.12 Pimprinaphine R= $CH_2C_6H_5$

2.13 Laboradorin 1 R= $CH_2CH(CH_3)_2$

2.14 Laboradorin 2 R= $CH_2CH_2CH_2CH_2CH_2CH_3$

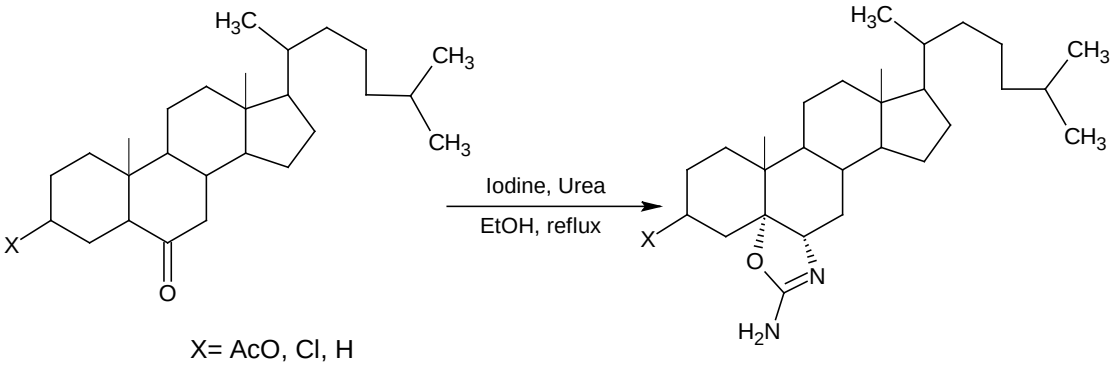


Indolyl-1,3,4-oxadiazoles

(2.15)

Shamsuzzaman et al., reported the synthesis of 6,5 fused steroidal oxazoles in cholestane series **(2.16)** [15]. The synthesis involves the reaction of cholestan-6-ones

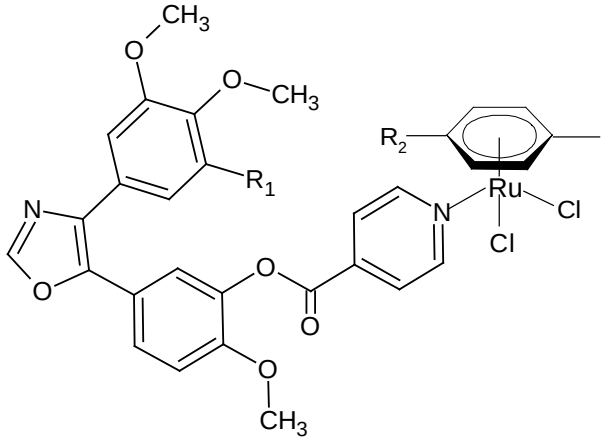
with urea and iodine.. Some of the compounds showed inhibitory action against both types of the bacteria (Gram-positive and Gram-negative) and five strains of fungi.



X= AcO, Cl, H

(2.16)

Bernhard Biersack et al., reported the synthesis of Oxazole-bridged combretastatin A-4 analogues **(2.17)** with enhanced anti-tumour activity [16]. All the compounds inhibited the growth of human HL-60 leukaemia and 518A2 melanoma cells at picomolar concentrations, their activities in cells of human colon carcinomas HT-29, which is CA-4-refractory, and HCT-116 as wild-type and p53(-/-) mutant forms, were more discriminative and structure-dependent.



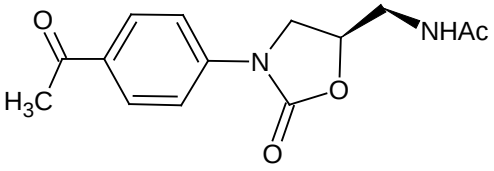
R_1= Cl, Br, OMe

R_2= H, *i*pr

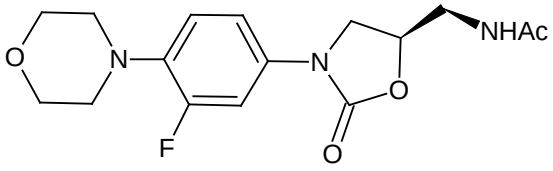
(2.17)

An effort to develop some novel drugs against some antibiotic resistant bacteria resulted in the discovery of oxazolidinone **(2.18)** (Dup-721) by DuPont, a new class

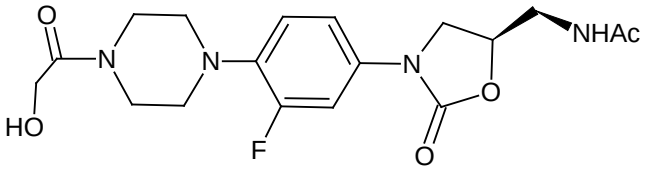
of synthetic antibacterial agent having activity against multidrug-resistant Gram-positive bacterial strains. [17] After a while, scientists at Pharmacia & Upjohn reported the development of linezolid **(2.19)** and eperezolid **(2.20)** which are currently under clinical trials. [18, 19] and now the optimization of the activity of oxazolidinones became a hot topic in the antibiotics research area, and various heterocycles are being introduced [20-22].



(2.18) Dup-721



(2.19) Linezolid

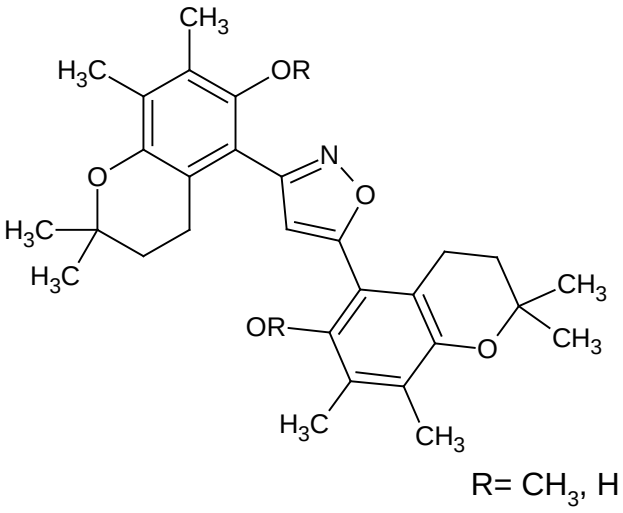


(2.20) Eperezolid

A.N. Pae et al, reported that an isoxazolyl substituent could enhance largely the activity of cephalosporins especially against Gram-positive bacteria, [23] Based on the findings, a positive effect of an isoxazole group on the activity of oxazolidinone was anticipated.

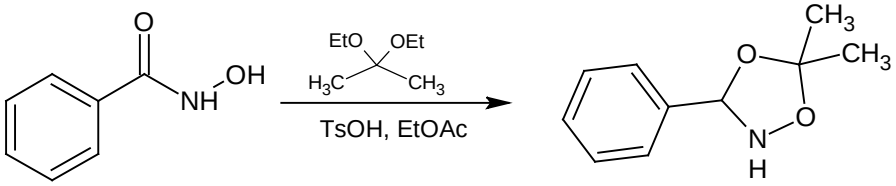
Maria Koufaki et al., reported the synthesis of 3- and 5-substituted (chroman-5-yl)-isoxazoles and (chroman-2-yl)-isoxazoles **(2.21)** using the copper(I)-catalysed cycloaddition reaction between *in situ* generated nitrile oxides and terminal acetylenes [24]. The activity of these compounds against oxidative stress-induced death (oxytosis) of neuronal HT22 cells was evaluated and interesting SARs for this group of analogues were derived. The vast majority of new chroman analogues displayed

high *in vitro* neuroprotective activity displaying EC_{50} values below 1 µM and lacked cytotoxicity.



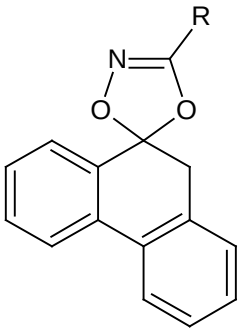
(2.21)

Dioxazoles, an important member of this family has received much attention due to its antiprotozoal properties. Dioxazolines are generally produced from the 1,3 cyclo addition reaction of nitrile N-oxides [25] with aldehydes and ketones *via* the corresponding oximes, [26] imidoyl chlorides, [27] nitro, [28] and furoxanes [29]. They can also be prepared by photolysis of acyl azides in the presence of ketones, [30] addition of hydroxamic acids onto acetylenic esters [31] and furans, [32] and observed in the rearrangement of N-acyloxaziridines [33]. Mukaiyama has reported the preparation of aryl derivatives through trans-acetalization [34]. Attempts have also been made to convert benzohydroxamic acid to the dioxazole **(2.22)** under a wide variety of reaction conditions, including the original Mukaiyama procedure.



(2.22)

Awad et al., reported the synthesis of 9(10)-phenanthrone- l0(9)-spiro-5'-aryl- 1 ',3',4'-dioxazoles **(2.23)**, through a 1,3-Dipolar cycloaddition of nitrile oxides. The dioxazoles were stable to both acids and alkalies [35].



R= C_6H_4Cl, $C_6H_3(OCH_3)Cl$

(2.23)

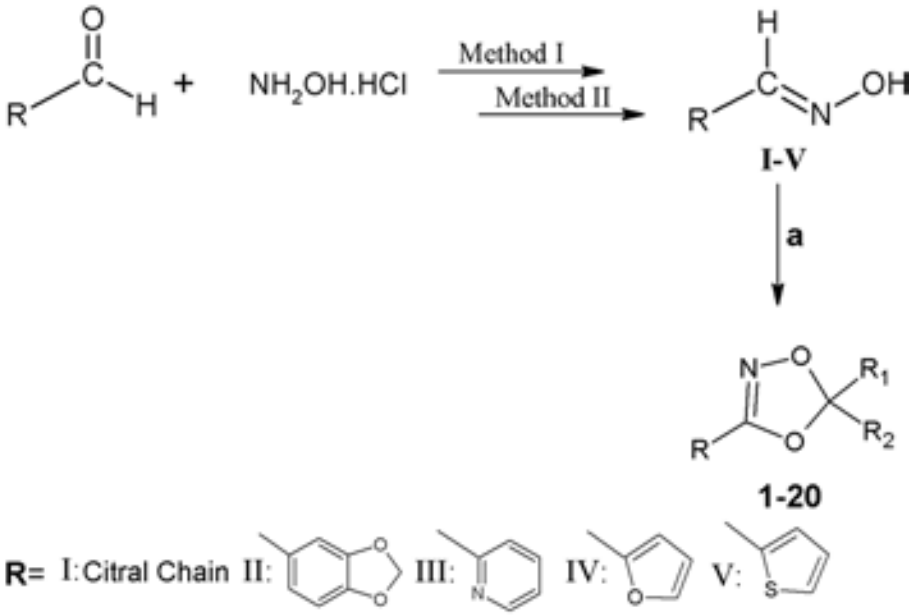
Bhat et al., reported some new derivatives of 3,5-substituted-1,4,2-dioxazoles with antiamoebic activity [36]. Dioxazole derivatives were synthesized in two steps via their corresponding oximes. In another similar study Iqbal et al., report synthesis of some Bis-dioxazole derivatives as potential antiamoebic agents [37]. Recently Irfan et al., reported some new dioxazole derivatives *via* the reaction of aldo-oxime in the presence of sodium hypochlorite and triethylamine in dichloromethane [38]. The effect of diaoxazoles on the inhibition of growth of *Entamoeba histolytica* and *Giardia intestinalis* in *vitro* has been determined.

This chapter discusses the synthesis of a series of terpene based 3,5-substituted-1,4,2-dioxazole derivatives and their antiamoebic activity.

2.2. RESULTS AND DISCUSSION

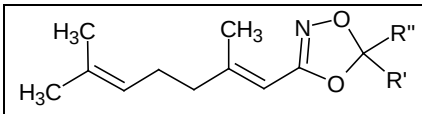
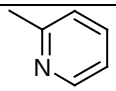
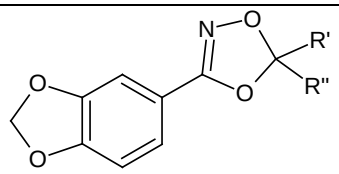
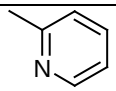
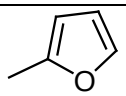
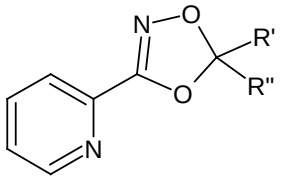
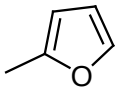
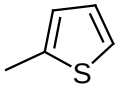
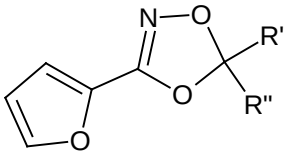
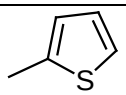
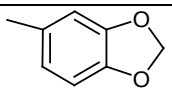
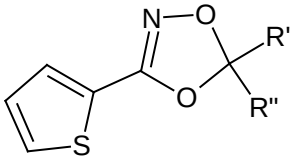
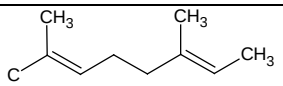
2.2.1. Chemistry

The outline synthesis of dioxazole derivatives (1-20) is given in Scheme 2.1. The reaction of corresponding oximes (I-V) with different aromatic aldehydes or ketones in ethylacetate using sodium hypochlorite and triethylamine yielded the respective dioxazole derivatives. The compounds in solid state showed sharp melting points and the elemental analysis was found in accordance with ± 0.3%. The compounds were stable and were soluble in DMSO, methanol, and chloroform. These terpene based dioxazole derivatives 1-20 were recrystallized from appropriate solvents, and final yield of 25-60% was obtained. The dioxazole derivatives were characterized by electronic, IR, ^{1}H, ^{13}C NMR, and mass spectroscopy.



Scheme 1: Synthesis of dioxazole derivatives (1-20).

Reagents and Conditions: *Method I: Pyridine, C_2H_5OH, reflux 24 h. Method II: H_2O, $NaHCO_3$, r.t.* ***(a)*** *aq. NaOCl, Et_3N, EtOAc, (R'-CO-R"),*

	Compound	R’	R”
 **(1-7)**	**1, 8**	CH_3	
 **(8-14)**	**2, 9**	H	
	3, 10	CH_3	
 **(15-16)**	**4, 11**	H	
	5, 12	CH_3	
 **(17-18)**	**6, 13**	H	
	7, 16, 18, 20	H	
 **(19-20)**	**14, 15, 17, 19**	H	

Characteristic IR bands provide significant indications for the formation of the oximes I-V and dioxazoles 1-20. The absence of a band at/or around 2665 cm^{-1} due to aldehydic proton and the appearance of characteristic bands at 3245-3260 cm^{-1} and 1622-1635 cm^{-1} due to ν(NO-H) and ν(C=N) respectively, confirmed the formation of the oximes I-V. The absence of a band at 3245-3260 cm^{-1} in all the dioxazoles 1-20 confirmed the conversion of the corresponding oximes into their respective dioxazoles. The ν(C=N) band in dioxazoles was in the range of 1642-1660 cm^{-1} and a new band in the range of 1120-1185 cm^{-1} arised due to (C-O-C) group, however, in some cases this band splited. In addition to these the representative bands due to carbon-carbon stretching of aliphatic and aromatic groups were also present. The

electronic spectra of the compounds 1-20 studied in the UV region, exhibited three absorption bands at 314-354 nm, 295-314 nm and 250-295 nm assigned to $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ transitions respectively. The band at 314-354 nm was assigned to the transition involving the azomethine group (C=N). The other two absorption bands at 295-314 nm and 250-295 nm were due to $\pi \rightarrow \pi^*$ transition of dioxazole ring and $n \rightarrow \sigma^*$ transition of azomethine nitrogen respectively.

The structure of the oximes I-V was further confirmed by ^{1}H NMR spectra. A singlet at 6.90-7.90 ppm due to (CH=N) proton showed condensation between carbonyl group of aldehydes and amino group of hydroxylamine hydrochloride. The signal at 9.43-11.13 ppm due to (N-OH) proton further confirmed the formation of the oximes. The structure of oximes I-V was further supported by ^{13}C NMR spectra. The absence of aldehydic carbon signal at 192 ppm and the presence of a signal at 146.0-149.80 ppm due to (C=N) confirmed the formation of oximes. The formation of dioxazoles was supported by the absence of a signal at 9.43-11.13 ppm and 6.90-7.80 due to (N-OH) and (N=CH) respectively in all the compounds 1-20. The singlet at 5.65-6.96 ppm, which arised due to (CH) group present at C-3 of the dioxazole ring, confirmed the condensation of oximes I-II with different hetero aromatic aldehydes/ketones and Terpene moieties and oximes III-V with different Terpene moieties. Methyl signals were obtained for compounds which contain methyl protons at C-3 of the dioxazole ring. For methyl group, a singlet at 1.90-2.54 ppm appeared in compounds 1, 3, 5, 8, 10 and 12. In addition, the signals for the different ring protons appeared in their respective range in all the compounds. ^{13}CNMR spectra further supported the confirmatory structures. The (C=N) signal around 155.1-169.6 ppm and (-OCO-) signal around 85.9-96.8 ppm clearly favoured the formation of dioxazole rings. The

signals due to the different hetero aromatic and aliphatic carbons resonate at their usual positions and the values are given in the experimental section.

2.2.2. *In vitro* antiamoebic activity

Preliminary experiments were carried out to determine the in vitro antiamoebic activity of all the compounds 1-20 by microdilution method using HM1:IMSS strain of *E. histolytica*. The results are summarized in Table 2.1. The data is presented in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic effect was compared with the most widely used antiamoebic medication metronidazole with 50% inhibitory concentration (IC_{50}) of 1.45 μM in our experiments. Since the target compounds were designed in three different ways based on the substituted molecules at position-3 and 5 of the dioxazole ring. The compounds (1-7), (8-14) and (15-20) showed activity in the range of IC_{50}= 1.40-3.01 μM, IC_{50}= 1.00-3.50 μM and IC_{50}= 1.05-3.05 μM respectively. The results showed that the compound 8 (IC_{50} =1.00 μM), 9 (IC_{50} = 1.03 μM), 10 (IC_{50} = 1.10 μM), 11 (IC_{50} = 1.09 μM), 16 (IC_{50}= 1.06 μM) and 18 (IC_{50} = 1.05 μM) *ex*hibited better antiamoebic activity than the standard drug metronidazole while compounds 7 & 14 were moderately active. Based on IC_{50} values, six compounds 8, 9, 10, 11, 16 & 18 were more active than the standard drug metronidazole. From the above results it can be inferred that most of the new compounds having piperonal ring skeleton in conjugation with pyridine ring or furan ring in the same compound showed significant antiamoebic activity and the modification of functionality produced significant change of activity. Thus, in the dioxazole series, antiamoebic activity can be positively modulated through the introduction of pyridine, furan and piperonal ring residue on the dioxazole ring. The

results were also statistically evaluated by analysis of variance. The null hypothesis was tested using t-test. The significativity of the difference between the IC_{50} values of metronidazole and the compounds 8, 9, 10, 11, 16 and 18 was evaluated by t-test. The values of the calculated T were found higher than the Table value of T at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

Table 2.1: *In vitro* antiamoebic activity of compounds (1-20) against HM1: IMSS strain of *E. histolytica* and toxicity profile. The compounds with bold font IC_{50} values are more active than metronidazole.

Compound	**Antiamoebic activity**		**Toxicity Profile**	
	IC_{50}(µM)	**S.D[a]. (±)**	**IC_{50} (µM)**	**SafetyIndex (SI)**
1	2.25	0.62	N.D	N.C
2	2.98	0.13	N.D	N.C
3	2.02	0.46	N.D	N.C
4	2.42	0.27	N.D	N.C
5	3.01	0.29	N.D	N.C
6	2.90	0.16	N.D	N.C
7	1.48	0.18	N.D	N.C
8	**1.00**	0.25	>200	>200
9	**1.03**	0.28	40	38.83
10	**1.10**	0.14	>200	>181.81
11	**1.09**	0.13	>200	>183.48
12	2.68	0.20	N.D	N.C
13	3.50	0.36	N.D	N.C
14	1.39	0.39	N.D	N.C
15	2.22	0.22	N.D	N.C
16	**1.06**	0.43	85	>80.18
17	2.78	0.29	N.D	N.C
18	**1.05**	0.31	200	190.47
19	3.05	0.19	N.D	N.C
20	2.45	0.26	N.D	N.C
MNZ	1.45	0.33	>200	>137.90

MNZ (Metronidazole)

2.2.3. Cytotoxicity studies (MTT Assay)

To ensure the toxicity of the most active compounds 8, 9, 10, 11, 16 and 18, they were tested against H9c2 cardiac myoblasts. A sub-confluent population of H9c2 cells was treated with increasing concentrations of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13-200 μM. Figure 2.24 depicts that all the compounds including the reference compound metronidazole showed a viability of 100% at the concentration range of 3.13 μM. Figure 2.24 depicts that the compounds **8**, **9**, **10**, **11**, **16** and **18** and metronidazole exhibited ≥82% viability at the concentration range of 3.13-25 μM. On increasing the concentration range up to 50, 100 and 200 μM the compounds showed moderate to high cytotoxicity against the *H9c2 cardiac myoblasts*. Compound 8 showed least cytotoxicity among all the compounds screened, with a remarkable viability of 88% at a concentration of 200 μM. The cytotoxicity IC_{50} values along with the standard deviation values of the compounds and metronidazle are given in Table 2.1. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Table 2.1. Compound 8 showed higher safety index value. Compound 10, 11 and 18 shows safety index value better than metronidazole. These results showed that further studies of these compounds can give a better lead in the drug field.

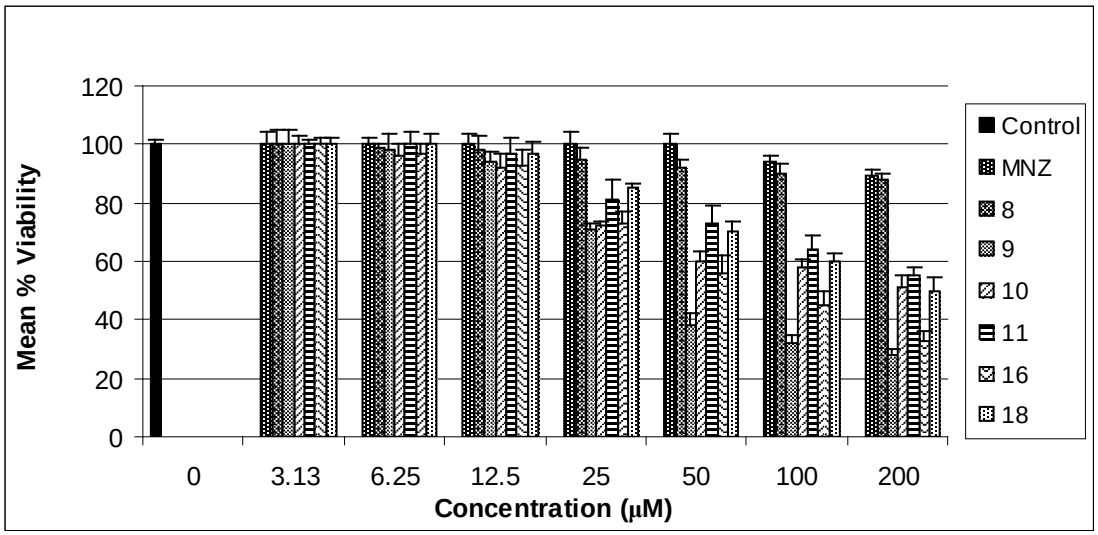


Figure 2.24: Percentage of viable cells after 48 h pre-treatment of H9c2 myoblasts with Metronidazole, compounds 8, 9, 10, 11, 16 and 18, evaluated by MTT assay.

2.3. EXPERIMENTAL

2.3.1. Synthesis

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results are uncorrected. Precoated aluminium sheets (silica gel 60 F_{254}, Merck Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Elemental analyses were performed on Heraeus Vario EL III analyzer at Central Drug Research Institute, Lucknow, India. Electronic spectra were recorded on a Shimadzu UV 1601 PC UV-Visible spectrophotometer. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 spectrometer using DMSO-d_6 as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer.

2.3.1.1. General procedure for the synthesis of oximes **(I-V)**

The oximes were synthesized by two methods (I & II). Although both methods resulted in good yields, however the method II is favoured, because of higher yield and less reaction time (Table 2.2).

2.3.1.1.1. Method I

An aldehyde (1 mmol) and hydroxylamine hydrochloride (1.25 mmol) in a solution of ethanol and pyridine (2:1) was refluxed with stirring for 24 hrs. After cooling, the mixture was concentrated and then poured into 600 ml of ice cold water. The solid mass was collected, washed with water, and crystallized from methanol to gave the

corresponding oxime. However in case of citral oxime an oil layer was formed which was extracted with ether and separated. The organic layer was dried with $NaSO_4$ and concentrated under reduced pressure.

2.3.1.1.2. Method II

To a solution of aldehyde (1 eq) and hydroxylamine hydrochloride (1.25 eq) in water (5 ml), a solution of sodium bicarbonate (1.25 eq) in water (10 ml) was added gradually with stirring, and the mixture was stirred for further 2-5 hours with the formation of a solid precipitate, which was filtered and dried. However in case of citral oxime (I), an organic layer was formed, which was separated and the aqueous layer was extracted with ether and added to the organic layer. The organic layer was dried with $NaSO_4$ and evaporated.

(1E,2E)-N-hydroxy-3,7-dimethylocta-2,6-dien-1-imine **(I):** Oil; Yield 92%; Anal. Calc. For $C_{10}H_{17}NO$: C 71.81, H 10.25, N 8.37%; found: C 71.79, H 10.26, N 8.39%; IR $\nu_{max}cm^{-1}$: 3250 (NO-H), 2864 (C-H), 1632 (C=N), 936 (N-O stretch); ^{1}H NMR (DMSO-d6) δ(ppm): 9.43 (broad s, 1H, N-OH), 7.32 (s, 1H, CH=N-OH), 5.90 (d, 1H, =CH-CH=N, J= 11.7 Hz), 5.08 (s, 1H, =CH-CH_2), 2.30-2.03 (m, 4H, CH_2-CH_2), 1.83 (s, 6H, CH_3), 1.68 (s, 3H, CH_3); $^{13}CNMR$ (DMSO-d_6) δ(ppm): 147.58 (C=N), ESI-MS m/z: [M^++1] 167.13

N-[(E)-1,3-benzodioxol-5-ylmethylidene]methanamine **(II):** White; Yield 90%; mp. 100-105^{0}C; Anal.Calc. For $C_8H_7NO_3$: C 58.18, H 4.27, N 8.48%. found: C 58.34, H 4.36, N 8.49%; IR $\nu_{max}cm^{-1}$: 3245 (NO-H), 2921 (C-H), 1635 (C=N), 937 (N-O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 10.85 (broad s, 1H, N-OH), 7.90 (s, 1H, CH=N-OH), 7.30-6.50 (m, 3H, Ar-H), 5.90 (s, -O-CH_2-O-); $^{13}CNMR$ (DMSO-d_6) δ(ppm): 148.10 (C=N), 101.6 (-O-CH_2-O); ESI-MS m/z: [M^++1] 166.04.

(E)-N-hydroxy-1-(pyridin-3-yl)methanimine **(III):** Pink; Yield 83%; mp. 118-120^{0}C; Anal.Calc. For $C_6H_6N_2O$: C 59.01, H 4.95, N 22.94, O 13.10 %. found: C 58.95, H 4.96, N 23.09, O 13.02 %; IR $\nu_{max}cm^{-1}$: 3260 (NO-H), 2920 (C-H), 1634 (C=N), 1630 (C=N), 937 (N-O stretch); ^{1}H NMR (DMSO-d6) δ(ppm): 10.76 (broad s, 1H, N-OH), 8.60 (d, 1H, py-H, J= 7.4 Hz), 8.45 (d, 1H, py-H, J= 7.2 Hz), 8.03-8.11 (dd, 1H, py-H, J= 8.7 Hz), 7.90-8.02 (dd, 1H, py-H, J= 8.4 Hz), 6.97 (s, 1H, CH=N-OH); ^{13}C NMR (DMSO-d_6) δ(ppm): 151.60 (C=N), 146.10 (C=N), 101.6 (-O-CH_2-O); ESI-MS m/z: [M^++1] 122.04

(E)-1-(furan-2-yl)-N-hydroxymethanimine **(IV):** White; Yield 80%; mp. 112-115^{0}C; Anal.Calc. For $C_5H_5NO_2$: C 47.23, H 3.96, N 11.01%. found: C 48.05, H 4.06, N 11.09%; IR $\nu_{max}cm^{-1}$: 3254 (NO-H), 3132 (C-H), 1628 (C=N), 935 (N-O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.13 (broad s, 1H, N-OH), 6.90 (s, 1H, CH=N-OH), 7.20 (d, 1H, furan ring-H, J= 7.6 Hz) 6.01 (dd 2H furan ring-H, J= 8.5, 8.9 Hz); ^{13}C NMR (DMSO-d_6) δ(ppm): 146.0 (C=N); ESI-MS m/z: [M^++1] 111.03

(E)-N-hydroxy-1-(thiophen-2-yl)methanimine **(V):** White; Yield 87%; mp. 98-101 ^{0}C; Anal.Calc. For C_5H_5NOS: C 47.23, H 3.96, N 11.01%. found: C 48.05, H 4.06, N 11.09%; IR $\nu_{max}cm^{-1}$: 3256 (NO-H), 3064 (C-H), 1622 (C=N), 942 (N-O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.05 (broad s, 1H, N-OH), 7.80 (s, 1H, CH=N-OH), 7.10-7.00 (dd, 2H, Thp-H, J= 7.8, 8.0 Hz), 6.80 (d, 1H, Thp-H, J= 5.4 Hz); ^{13}C NMR (DMSO-d_6) δ(ppm): 149.8 (C=N); ESI-MS m/z: [M^++1] 128.03.

Table 2.2: Synthesis of oximes I-V by method I &II.

Oximes	Method I (Yield)	Reaction time	Method II (Yield)	Reaction time
I	76%	24 hr	92%	2 hr
II	80%	24 hr	90%	3-5 hr
III	70%	24 hr	83%	2-3 hr
IV	70%	24 hr	80%	3 hr
V	72%	24 hr	87%	3-4 hr

2.3.1.2. General procedure for the synthesis of dioxazoles (1-20)

A 13% aqueous solution of NaOCl (1.6 equiv.) was added to a solution of aldehydes (1 equiv.) and triethylamine (0.1 equiv.) in ethyl acetate under argon atmosphere. The oxime (1 equiv.) in ethyl acetate was added dropwise (over a period of 1 h) at 0°C to the above solution and stirred at room temperature for 12-15 hrs and refluxed for additional 10-12 hrs, depending upon the reaction time (monitored by TLC). The reaction mixture was cooled to room temperature and water was added to it. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with water, brine, dried over $NaSO_4$, filtered and concentrated under *vacuo*. The compounds were crystallized using dichloromethane-hexane solution.

***2-(5-methyl-3-((E)-2, 6-dimethylhepta-1, 5-dienyl)-1,4,2-dioxazole-5-yl) pyridine* (1):** Cream; Yield 25%; mp. 183-185 ^{0}C; Anal. Calc. For $C_{17}H_{22}N_2O_2$: C 71.30, H 7.74, N 9.78%; found: C 71.27, H 7.70, N 9.79%; UV λ_{max}nm: 352, 311, 250; IR ν_{max}cm^{-1}: 3054 (C-H stretch py), 1652 (C=N), 1645 (C=C stretch), 1615 (C=N, py); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.60 (d, 1H, py-H, J= 7.4 Hz), 8.40 (d, 1H, py-H, J= 7.2 Hz), 8.12 (dd, 1H, py-H, J=8.4, 8.5 Hz), 7.95 (dd, 1H, py-H, J= 7.8, 8.2 Hz), 5.86 (s,1H, =CH-C), 5.01 (s, 1H, =CH-CH_2), 2.57-2.61 (m, 4H, CH_2-CH_2), 2.17 (s, 3H,

CH_3 dioxazole ring), 1.99 (s, 3H, CH_3), 1.68 (s, 6H, CH_3), ^{13}C NMR (DMSO-d_6) δ(ppm):162.4 (C=N), 160.1 (C=N), 154.2, 133.5, 123.7, 40.5, 26.7, 25.1, 19.4, 18.1, (Aliphatic chain-C), 147.6, 134.4, 122.8, 120.6, (Ar-C), 95.1(O-C-O); ESI-MS m/z: $[M^+ +1]$ 287.17.

***2-(3-((E)-2,6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazole-5-yl)pyridine* (2):** Oil; Yield 30%; Anal. Calc. For $C_{16}H_{20}N_2O_2$: C 70.56, H 7.40, N 10.29%; found: C 70.51, H 7.48, N 10.35%; UV λ_{max}nm: 321, 299, 267; IR $\nu_{max}cm^{-1}$: 3064 (C-H stretch py), 1645 (C=N), 1660 (C=N)1642 (C=C stretch); ^1H NMR (DMSO-d_6) δ(ppm): 7.80 (dd, 1H, py-H), 7.71 (d, 1H, py-H, J= 7.4 Hz), 7.62 (d, 1H, py-H, J= 7.2 Hz),7.26 (dd, 1H, py-H), 6.91 (s, 1H, C-H (dioxazole ring)) 5.08 (s, 2H, -CH=C), 2.33-2.10 (m, 4H, CH_2-CH_2), 1.68 (s, 6H, CH_3), 1.55 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 155.1 (C=N), 158.5 (C=N py), 154.8, 134.8, 38.5, 25.7, 24.3, 20.4, 18.7, 18.6, (Aliphatic C) 149.3, 137.4, 123.9, 121.0, (Ar-C), 87.5(O-C-O); ESI-MS m/z: $[M^+ +1]$ 273.15.

***5-(furan-2-yl)-5-methyl-3-((E)-2,6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazole* (3):** Oil; Yield 25%; Anal. Calc. For $C_{16}H_{21}NO_3$: C 69.79, H 7.69, N 5.09%; found: C 69.76, H 7.70, N 5.11%; UV λ_{max}nm: 332, 298, 276; IR $\nu_{max}cm^{-1}$: 3130 (C-H stretch furan ring), 1632 (C=N) 1641 (C=C stretch);^1H NMR (DMSO-d_6) δ(ppm): 8.02 (d, 1H furan ring-H, J= 5.6 Hz) 6.25 (dd 2H furan ring-H, J= 8.7, 8.5 Hz), 5.86 (s,1H, =CH-C), 5.07 (s, 1H, =CH-CH_2), 2.61-2.57 (m, 4H, CH_2-CH_2), 2.54 (s, 3H, CH_3 dioxazole ring), 1.68 (s, 3H, CH_3), 1.53 (s, 6H, CH_3), ^{13}C NMR (DMSO-d_6) δ(ppm): 159.6 (C=N), 152.2, 134.8, 124.3, 41.2, 28.7, 26.6, 19.5, 18.3 (Aliphatic chain-C) 155.6, 140.2, 110.5, 106.4 (Ar-C) 96.8 (O-C-O); ESI-MS m/z: $[M^+ +1]$ 276.15.

***5-(furan-2-yl)-3-((E)-2, 6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazole* (4):** Oil; Yield 50%; Anal. Calc. For $C_{15}H_{19}NO_3$: C 68.94, H 7.33, N 5.36%; found: C 69.14, H 7.28,

N 5.37%; UV λ_{max}nm: 336, 312, 286; IR $\nu_{max}cm^{-1}$: 3030 (C-H stretch furan ring), 1648 (C=N), 1643 (C=C stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.08 (d, 1H furan ring-H, J= 5.2 Hz), 6.52 (dd 2H furan ring-H, J= 5.6, 5,6 Hz), 6.16 (dd 2H furan ring-H, J= 5.8, 5,4 Hz), 5.90 (s, 1 H, C-H (dioxazole ring), 5.08 (s, 2H, =CH-CH_2), 2.25-2.05 (m, 4H, CH_2-CH_2), 1.83 (s, 3H, CH_3) 1.50 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.4 (C=N), 162.4 (C=N)122.6, 154.3, 137.8, 40.7, 28.1, 25.3, 20.5, 17.8 (Aliphatic chain-C) 151.6, 138.4, 111.7, 106.7, (Ar-C)), 90.7(O-C-O); ESI-MS m/z: [M^{+}+1] 262.14.

5-methyl-3-((E)-2,6-dimethylhepta-1,5-dienyl)-5-(thiophenyl-2-yl)-1,4,2-dioxazole **(5):** Oil; Yield 40%; Anal. Calc. For $C_{16}H_{21}NO_2S$: C 65.95, H 7.26, N 4.81%; found: C 65.84, H 7.28, N 4.87%; UV λ_{max}nm: 344, 304, 281; IR $\nu_{max}cm^{-1}$: 3125 (C-H stretch thiophene ring), 1652 (C=N),1640 (C=C stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (dd 1H Thp-H, J= 8.6, 8.4 Hz), 7.74 (d, 1H Thp-H, J= 7.6 Hz), 7.13 (d, 1H Thp-H, J= 7.6 Hz), 6.08 (s, 1H, =CH-C), 5.08 (s, 1H, =CH-CH_2), 2.54 (s, 3H, CH_3 dioxazole ring), 2.27-2.13 (m, 4H, CH_2-CH_2), 1.38 (s, 6H, CH_3), 1.35 (s, 3H, CH_3), ^{13}C NMR (DMSO-d_6) δ(ppm): 160.9 (C=N), 149.0, 133.0, 123.5, 36.5, 30.1, 21.6, 21.6, 16.9, (Aliphatic chain-C) 153.2, 135.5, 105.6, 102.5, (Ar-C), 95.0 (O-C-O); ESI-MS m/z: [M^{+}+1] 292.13.

3-((E)-2,6-dimethylhepta- 1,5-dienyl)- 5-(thiophen-2-yl)-1,4,2-dioxazole **(6):** Oil; Yield 30%; Anal. Calc. For $C_{15}H_{19}NO_2S$: C 64.95, H 6.90, N 5.05%; found: C 65.04, H 6.98, N 5.05%; UV λ_{max}nm: 319, 299, 261; IR $\nu_{max}cm^{-1}$: 3095 (C-H stretch thiophene ring), 1660 (C=N), 1644 (C=C stretch), ^{1}H NMR (DMSO-d_6) δ(ppm): 7.74 (dd 1H Thp-H) 7.53 (d, 1H Thp-H, J= 5.6 Hz), 7.43 (d, 1H Thp-H, J= 5.6 Hz), 6.30 (s, 1H, C-H (dioxazole ring), 5.90 (s, 1H, =CH-C), 5.10 (s, 1H, =CH-CH_2), 2.25-2.13

(m, 4H, CH_2-CH_2), 1.38 (s, 6H, CH_3), 1.35 (s, 3H, CH_3), ^{13}C NMR (DMSO-d_6) δ(ppm): 166.5 (C=N), 153.0, 135.0, 121.2, 34.5, 32.5, 25.6, 20.5, 17.0; (Aliphatic chain-C) 153.2, 135.5, 105.6, 102.5, (Ar-C), 89.5 (O-C-O); ESI-MS m/z: [M^++1] 278.17.

5-(benzo[d][1,3]dioxol-5-yl)-3-((E)-2,6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazole **(7):** White; Yield 40%; mp. 123-125^{0}C; Anal. Calc. For $C_{18}H_{21}NO_4$: C 68.55, H 6.71, N 4.44 %; found: C 68.54, H 6.78, N 4.40%; UV λ_{max}nm: 354, 314, 271; IR ν_{max}cm^{-1}: 1647 (C=N), 1645 (C=C stretch), ^{1}H NMR (DMSO-d_6) δ(ppm): 7.80-7.56 (m, 3H, Ar-H), 6.96 (s, 1H, C-H (dioxazole ring), 6.16 (s -O-CH_2-O-), 5.43 (s, 1H, =CH-CH_2), 4.75 (s, 1H, =CH-C), 2.14-2.03 (m, 4H, CH_2-CH_2), 1.90 (s, 6H, CH_3), 1.38 (s, 3H, CH_3);^{13}C NMR (DMSO-d_6) δ(ppm): 162.4 (C=N), 150.5, 134.3, 120.5, 34.9, 30.5, 23.5, 22.6, 17.5, (Aliphatic chain-C) 145.6, 147.5, 120.5, 114.6, 100.5, (Ar-C) 89.7(O-C-O); ESI-MS m/z: [M^++1] 316.18.

2-(3-(benzo[d][1, 3]dioxol-5-yl)-5-methyl-1,4,2-dioxazol-5-yl)pyridine **(8):** Cream; Yield 60%; mp. 140-145^{0}C; Anal. calc. for $C_{15}H_{12}N_2O_4$: C 63.38, H 4.25, N 9.85%; found: C 63.45, H 4.30, N 9.96%; UV λ_{max}nm: 340, 301, 265; IR ν_{max}cm^{-1}: 3054 (Ar-H), 3020 (C-H), 1660 (C=N), 1650 (C=N py); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (d, 1H, py-H, J= 7.2 Hz), 7.71 (d, 1H, py-H, J= 7.2 Hz), 7.60 (dd, 1H, py-H, J= 7.8, 8.2 Hz), 7.34 (dd, 1H, py-H, J= 8.2, 8.6 Hz), 6.92-6.85 (m, 3H, Ar-H), 6.07 (s, 2H, -O-CH_2-O), 1.90 (s, 3H, CH_3 dioxazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.5 (C=N), 150.6, 146.2, 145.5, 123.4, 115.7, 113.0, (Ar-C), 101.6 (-O-CH_2-O), 94.8 (O-C-O), 27.8 (CH_3); ESI-MS m/z: [M^++1] 285.10.

2-(3-(benzo[d][1,3]dioxol-5-yl)-1,4,2-dioxazol-5-yl)pyridine **(9):** Cream; Yield 27%; mp. 130-134^{0}C; Anal. calc. for $C_{14}H_{10}N_2O_4$: C 62.22, H 3.73, N 10.37%; found: C

63.01, H 3.83, N 10.26%; UV λ_{max}nm: 346, 307, 255; IR $\nu_{max}cm^{-1}$: 3065 (C-H), 3025 (Ar-H), 1656 (C=N), 1643 (C=N py); ^{1}H NMR (DMSO-d_6) δ(ppm):): 8.81 (d, 1H, py-H, J= 7.6 Hz), 8.33 (d, 1H, py-H, J= 7.5 Hz), 8.02 (dd, 1H, py-H, J= 8.6, 8.4 Hz), 7.71 (dd, 1H, py-H, J= 8.2, 8.4 Hz), 6.91-7.59 (m, 3H, Ar-H), 6.52 (s, 1H, C-H (dioxazole ring), 5.94 (s, 2H, -O-CH_2-O-);^{13}C NMR (DMSO-d_6) δ(ppm): 156.4 (C=N), 159.8 (C=N py), 152.5, 150.2, 146.5, 122.5, 114.7, 112.5 (Ar-C), 103.5 (-O-CH_2-O), 87.5 (O-C-O); ESI-MS m/z: [M^++1] 271.08.

3-(benzo[d][1,3]dioxol-5-yl)-5-(furan-2-yl)-5-methyl-1,4,2-dioxazole **(10):** Cream; Yield 29%; mp. 150-155^{0}C; Anal. calc. for $C_{14}H_{11}NO_5$: C 61.54, H 4.06, N 5.13%; found: C 61.63, H 3.93, N 5.23%; UV λ_{max}nm: 338, 306, 275; IR $\nu_{max}cm^{-1}$: 3072 (Ar-H), 3045 (C-H), 1650 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (d, 1H, furan ring-H, J= 5.6 Hz) 7.51-7.31 (m, 3H Ar-H), 7.25 (d, 1H, furan ring–H, J= 5.4 Hz), 7.13 (dd, 1H, furan ring-H, J= 7.6, 7.6 Hz), 5.86 (s, 2H, -O-CH_2-O-), 1.90 (s, 3H, dioxazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 162.4 (C=N), 150.4, 148.1, 141.4, 123.7, 128.5, 114.5, 110.5, 104.0 (Ar-C), 91.5 (O-C-O), 25.6 (CH_3); ESI-MS m/z: [M^++1] 274.06.

3-(benzo[d][1,3]dioxol-5-yl)-5-(furan-2-yl)-1,4,2-dioxazole **(11):** Brown; Yield 40%; mp. 153-155^{0}C; Anal. calc. for $C_{13}H_9NO_5$: C 60.24, H 3.50, N 5.40%; found: C 60.58, H 3.42, N 5.33%; UV λ_{max}nm: 354, 312, 265; IR $\nu_{max}cm^{-1}$: 3061 (Ar-H), 3027 (C-H), 1638 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.80 (d, 1H furan ring-H, J= 5.7 Hz), 7.74-7.60 (m, 3H, Ar-H), 7.26 (dd 1H furan ring-H, J= 5.6, 5.8 Hz), 7.13 (d, 1H furan ring-H, J= 5.6 Hz), 6.14 (s -O-CH_2-O-) 6.08 (s, 1H, C-H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ(ppm): 157.6 (C=N), 150.4, 148.1, 141.4, 128.5, 123.7, 114.5, 110.5, 104.0, (Ar-C) 87.5 (O-C-O); ESI-MS m/z: [M^++1] 260.04.

3-(benzo[d][1,3]dioxol-5-yl)-5-methyl-5-(thiophen-2-yl)-1,4,2-dioxazole **(12):**

Yellowish; Yield 50%; mp. 120-123^{0}C; Anal. calc. for $C_{14}H_{11}NO_4S$: C 58.12, H 3.83, N 4.84%; found: C 58.34, H 3.73, N 4.84%; UV λ_{max}nm: 330, 311, 291; IR ν_{max}cm^{-1}: 3100 (Ar-H), 3065 (C-H), 1656 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.80 (dd, 1H, Thp-H, J= 5.8, 5.6 Hz), 7.71-7.56 (m, 3H, Ar-H), 7.26 (d, 1H Thp-H, J= 7.8 Hz), 6.91 (d, 1H Thp-H, J= 7.4 Hz), 6.09 (s, 2H, -O-CH_2-O-), 2.19 (s, 3H, dioxazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 157.4 (C=N), 156.4, 150.6, 148.3, 147.5, 125.5, 112.2, 108.5, 102.5 (Ar-C), 92.5 (O-C-O), 28.5; ESI-MS m/z: [M^++1] 290.04.

3-(benzo[d][1,3]dioxol-5-yl)-5-(thiophen-2-yl)-1,4,2-dioxazole **(13):** Oil; Yield 52%; Anal. calc. for $C_{13}H_9NO_4S$: C 56.72, H 3.30, N 5.09%; found: C 56.80, H 3.40, N 5.23%; UV λ_{max}nm: 325, 297, 255; IR ν_{max}cm^{-1}: 3055 (C-H), 3034 (Ar-H), 1640 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (dd, 1H, Thp-H, J= 5.6, 5.8 Hz), 7.26-7.13 (m, 3H, Ar-H), 6.92 (d, 1H, Thp-H, J= 7.2 Hz), 6.80 (d, 1H, Thp-H, J= 7.4 Hz), 6.04 (s, 1H, C-H (dioxazole ring)), 5.70 (s, 2H, -O-CH_2-O-); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.1 (C=N), 103.2, 158.5, 152.6, 147.5, 146.2, 127.5, 113.5, 109.2, (Ar-C) 89.0 (O-C-O); ESI-MS m/z: [M^++1] 276.03.

3-(benzo[d][1,3]dioxol-6-yl)-5-((E)-2,6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazole **(14):** Grey; Yield 58%; mp. 145-147^{0}C; Anal. calc. for $C_{18}H_{21}NO_4$: C 68.55, H 6.71, N 4.44 %; found: C 68.32, H 6.73, N 4.33%; UV λ_{max}nm: 335, 298, 275; IR ν_{max}cm^{-1}: 3075 (C-H), 3065 (Ar-H), 1650 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.53-7.13 (m, 3H, Ar-H), 6.04 (s, 2H, -O-CH_2-O-) 5.88 (s, 1H, =CH-C), 5.65 (s, 1H, C-H (dioxazole ring)), 5.10 (s, 1H, =CH-CH_2), 2.26-2.13 (m, 4H, CH_2-CH_2), 1.38 (s, 3H, CH_3), 1.35 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 162.4 (C=N), 153.0, 135.0, 121.2, 32.5,

34.5, 25.6, 20.5, 17.0 (Aliphatic chain-C) 146.2, 127.5, 113.5, 109.2, 103.2 (Ar-C) 86.7 (O-C-O); ESI-MS m/z: [M^++1] 316.15.

2-(5-((E)-2, 6-dimethylhepta-1,5-dienyl)-1,4,2-dioxazol-3-yl)pyridine **(15):** Grey; Yield 60%; mp. 140-143^{0}C; Anal. calc. for $C_{16}H_{20}N_2O_2$: C 70.56, H 7.40, N 10.29%; found: C 70.64, H 7.33, N 10.24%; UV λ_{max}nm: 321, 303, 263; IR ν_{max}cm^{-1}: 1642 (C=C stretch), 3054 (C-H stretch py), 1650 (C=N); 1635 (C=N py); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.08 (d, 1H, py-H, J= 5.2 Hz), 7.73 (d, 1H, py-H, J= 5.6 Hz), 7.56 (dd, 1H, py-H), 7.26 (dd, 1H, py-H), 6.07 (s, 1H, C-H (dioxazole ring), 5.90 (s, 1H, =CH-C), 5.08 (s, 1H, =CH-CH_2), 2.61-2.29 (m, 4H, CH_2-CH_2), 1.99 (s, 3H, CH_3), 1.68 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 159.8 (C=N), 160.5 (C=N py), 151.8, 134.7, 120.5, 36.9, 34.5, 24.6, 22.8, 17.5, (Aliphatic chain-C) 149.6, 146.5, 136.9, 127.5, 123.5 (Ar-C) 87.5 (O-C-O); ESI-MS m/z: [M^++1] 273.15.

2- (5- (benzo[d][1, 3] dioxol-5-yl)-1,4,2-dioxazol-3-yl)pyridine **(16):** Yellowish; Yield 50%; mp. 135-137^{0}C; Anal. calc. for $C_{14}H_{11}NO_4S$: C 58.12, H 3.83, N 4.84%; found: C 58.34, H 3.73, N 4.84%; UV λ_{max}nm: 350, 313, 265; IR ν_{max}cm^{-1}: 3075 (C-H), 3054 (Ar-H), 1659 (C=N); 1630 (C=N py);^{1}H NMR (DMSO-d_6) δ(ppm): 8.50 (d, 1H, py-H, J= 7.6 Hz), 7.83 (d, 1H, py-H, J= 7.4 Hz), 7.74 (dd, 1H, py-H, J= 8.9, 8.6 Hz), 7.26 (dd, 1H, py-H, J= 8.4, 8.6 Hz), 6.92-6.85 (m, 3H, Ar-H), 6.04 (s, 1H, C-H (dioxazole ring)), 5.88 (s -O-CH_2-O-); ^{13}C NMR (DMSO-d_6) δ(ppm): 157.9 (C=N), 160.5 (C=N), 150.4, 143.2, 134.6, 128.5, 122.5, 112.9, 108.7, 102.5, (Ar-C), 93.7 (O-C-O); ESI-MS m/z: [M^++1] 271.06.

3-(furan-2-yl)-5-((E)-2, 6-dimethylhepta-1, 5-dienyl)-1,4,2-dioxazole **(17):** Oil; Yield 34%; Anal. calc. for $C_{15}H_{19}NO_3$: C 68.94, H 7.33, N 5.36 %; found: C 69.04, H 7.31, N 5.34 %; UV λ_{max}nm: 322, 296, 273; IR ν_{max}cm^{-1}: 1642 (C=C stretch), 3125

(C-H), 1643 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.81 (d, 1H furan ring-H, J= 5.6 Hz), 6.96 (dd, 2H furan ring –H, J= 5.8, 5.6 Hz) 5.86 (s, 1H, C-H (dioxazole ring)); 5.45 (s, 1H, =CH-C), 5.10 (s, 1H, =CH-CH_2), 2.61-2.54 (m, 4H, CH_2-CH_2), 1.99 (s, 3H, CH_3), 1.60 (s, 6H, CH_3), ^{13}C NMR (DMSO-d_6) δ(ppm): 162.4 (C=N), 17.5, 22.8, 24.6, 34.5, 36.9, 120.5, 134.7, 151.8 (Aliphatic chain-C) 105.6, 140.5 (Ar-C), 96.3 (O-C-O); ESI-MS m/z: [M^++1] 262.14.

5-(benzo[d][1,3]dioxol-5-yl)-3-(furan-2-yl)-1,4,2-dioxazole **(18):** Brown; Yield 32%; mp 145-147^{0}C; Anal. calc. for $C_{13}H_9NO_5$: C 60.24, H 3.50, N 5.40 %; found: C 60.14, H 3.51, N 5.44 %; UV λ_{max}nm: 327, 303, 278; IR ν_{max}cm^{-1}: 3065 (Ar-H), 3030 (C-H), 1660 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.99 (d, 1H furan ring-H, J= 7.2 Hz), 7.26-6.98 (m, 3H, Ar-H), 6.55 (dd, 2H, furan ring-H, J= 7.8, 7.4 Hz), 5.94 (s, 2H, -O-CH_2-O-), 5.90 (s, 1H, C-H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.0 (C=N), 98.5,108.2, 112.6, 117.4, 135.6, 147.9, 152.0 (Ar-C), 96.3 (O-C-O); ESI-MS m/z: [M^++1] 260.05.

5-((E)-2, 6-dimethylhepta-1,5-dienyl)-3-(thiophen-2-yl)-1,4,2-dioxazole **(19):** Oil; Yield 60%; Anal. calc. for $C_{15}H1_9NO_2S$: C 64.95, H 6.90, N 5.05 %; found: C 64.84, H 7.01, N 5.14 %; UV λ_{max}nm: 348, 310, 291; IR ν_{max}cm^{-1}: 1642 (C=C stretch), 3135 (C-H stretch thiophene ring), 3054 (Ar-H), 1639 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.04 (dd, 2H, Thp-H, J= 5.8, 5.1 Hz) 7.73 (d, 1H Thp-H, J= 7.2 Hz), 6.04 (s, 1H, C-H (dioxazole ring)), 5.86 (s, 1H, =CH-C), 5.07 (s, 1H, =CH-CH_2), 2.14-2.03 (m, 4H, CH_2-CH_2), 1.90 (s, 3H, CH_3), 1.60 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.5 (C=N), 17.8, 19.5, 27.4, 36.5, 40.5, 120.4, 131.5 (Aliphatic-C), 123.2, 130.6 (Ar-C), 87.3 (O-C-O); ESI-MS m/z: [M^++1] 278.11.

***5-(benzo[d][1,3]dioxol-5-yl)-3-(thiophen-2-yl)-1,4,2-dioxazole* (20):** Oil; Yield 50%; Anal. calc. for $C_{13}H1_9NO_4S$: C 56.72, H 3.30, N 5.09 %; found: C 64.84, H 7.01, N 5.14 %; UV λ_{max}nm: 318, 295, 250; IR $\nu_{max}cm^{-1}$: 3054 (Ar C-H), 3130 (C-H stretch thiophene ring) 1660 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (dd, 2H, Thp-H, J= 5.6, 5.8 Hz), 7.13 (d, 1H. Thp-H, J= 7.2 Hz), 6.92-6.85 (m, 3H, Ar-H), 6.02 (s, 2H, -O-CH_2-O-), 5.86 (s, 1H, C-H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ(ppm): 156.7 (C=N), 152.6, 149.9, 134.6, 122.0, 118.3, 114.6, 107.5, 99.7, (Ar-C), 85.9 (O-C-O); ESI-MS m/z: [M^++1] 276.03.

2.3.2. *In vitro* antiamoebic assay

All the compounds **1-20** were screened *in vitro* for antiamoebic activity against HM1: IMSS strain of *E. histolytica* by microdilution method [39]. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [40]. The test compounds (1 mg) were dissolved in DMSO (40 μL, level at which no inhibition of amoeba occurs) [41, 42]. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg mL^{-1}. Two-fold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). All the experiments were carried out in triplicate at each concentration level and repeated thrice. The amoeba suspension was prepared from a confluent culture by pouring off the medium at 37 ^{0}C and adding 5 ml of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The number of amoeba mL^{-1} was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The

suspension was diluted to 105 organism mL^{-1} by adding fresh medium and 170 μL of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 μL). An inoculum of 1.7 × 104 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 37^0C for 72 hrs. After incubation, the growth of amoeba in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. Plate was then immediately washed with sodium chloride solution (0.9%) at 37^0C. This procedure was completed quickly and the plate was not allowed to cool in order to prevent the detachment of amoebae. The plate was allowed to dry at room temperature and the amoebae were fixed with methanol and when dried, stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 μL portion of 0.1N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The % inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting line from which the IC_{50} value was found. The IC_{50} values in μM are reported in Table 2.1.

2.3.3. Cytotoxicity studies (MTT assay)

H9c2 rat cardiac myoblasts were cultured and maintained as monolayer in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal

bovine serum (heat inactivated), 100 units/ml penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37°C in humidified incubator with 5% CO_2 [43]. Only viable cells were used in the assay. Exponentially growing cells were plated at 1.2×10^4 cells per well into 96-well plates and incubated for 48 h before the addition of drugs to achieve the maximum confluency of the cells. Stock solutions were prepared by dissolving the compounds in 10% (v/v) DMSO and further diluted with fresh complete medium to achieve 1 M concentration. The growth-inhibitory effects of the compounds were measured using standard tetrazolium MTT assay. Cells were incubated with different concentrations of metronidazole and compounds 8, 9, 10, 11, 16 & 18 for 48 hours at 37 ^{0}C in 5% CO_2 humidified incubator together with untreated control sample. At appropriate time points, cells were washed in PBS, treated with 50 μL MTT solution (5 mg mL^{-1}, tetrazolium salt) and incubated for 4 hr at 37 ^{0}C. At the end of the incubation period, the medium was removed and pure DMSO 150 μL was added to each well. The metabolized MTT product dissolved in DMSO was quantified by measuring the absorbance at 570 nm on an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) with a reference wavelength of 655 nm. All assays were performed in triplicate and repeated thrice. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

2.4. CONCLUSION

The combination of extended synthetic analogues of natural molecules leads to discovery of chemical entities which might be excellent antiamoebic compounds as depicted in these results. 3, 5-substituted 1,4,2-dioxazole derivatives 1-20, bearing terpene moieties were synthesized and screened against HM1: IMSS strain of *E. histolytica*. Compound **8** (2-(3-(benzo[d][1, 3]dioxol-5-yl)-5-methyl-1,4,2-dioxazol-5-yl)pyridine) showed the most promising results based on antiamoebic screening and cytotoxicity studies. Being highly antiamoebic this compound can be explored in future as an option for decreasing pathogenic potential of infecting *E. histolytica* species.

2.5. REFERENCES

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Chapter 3

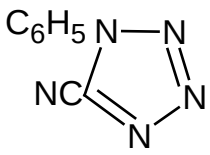
Synthesis, Characterization and Antiamoebic activity of tetrazole and triazine derivatives

3.1. INTRODUCTION

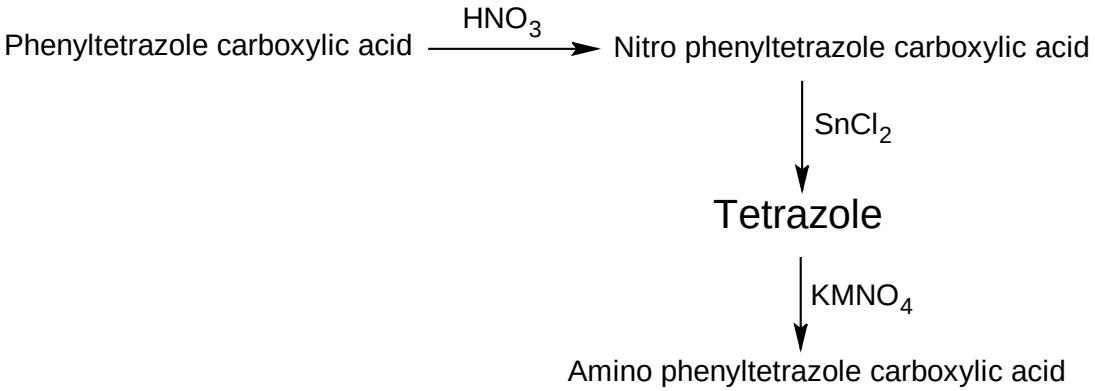
Tetrazoles are a class of synthetic organic heterocyclic compound, consisting of a 5-member ring of four nitrogen and one carbon atom (plus hydrogens). The simplest is tetrazole itself, CN_4H_2. They are unknown in nature. Among the stable structures, this heteroaromatic system contains the greatest number of nitrogen atoms, which is the reason why tetrazoles exhibit the extreme values of acidity, basicity, and complex formation constants. They have specific thermochemical properties and exhibit multiple reactivity [1-4]. The practical importance of these heterocycles results from a combination of the above-mentioned properties. The tetrazole ring is the fragment of a number of modern drugs (antibacterial, anti-allergic, anti-inflammatory, angiotensin II antagonists, etc.) [5–8]. On the basis of tetrazoles being highly effective explosives, rocket fuel and gas generating compositions have been developed [9-13]. The numerous possibilities of coordination of tetrazole ring with metal ions permits to use these compounds as effective complexones and as corrosion inhibitors [4,14–16]. The presence of several reaction centers and the possibility of prototropy in tetrazoles afford the conditions for their use in organic and bioorganic synthesis as reagents and catalysts [17-19]. Many of the above-mentioned properties of NH unsubstituted tetrazoles are related to their ability to act as acids and bases, and also to the possibility of prototropic annular tautomerism in the case of neutral molecules and conjugated acids [20, 21]. Tetrazoles exhibit potential biological activity because the tetrazole ring is considered a biomimic of the carboxylic acid functional group (Figure 3.1). However, the tetrazole moiety is metabolically more stable than the carboxylic acid group [22]. This special characteristic suggests that tetrazoles will continue to have a significant impact in pharmaceutical research.

Figure 3.1: *Structural comparison of carboxylic acid moiety and Tetrazole ring.*

The first tetrazole was prepared in **1885** by the Swedish Chemist, J. A. Bladin [23] observed that the action of nitrous acid on dicyanophenylhydrazine led to the formation of a compound, **$C_8H_5N_5$,** to which he ascribed the formula:

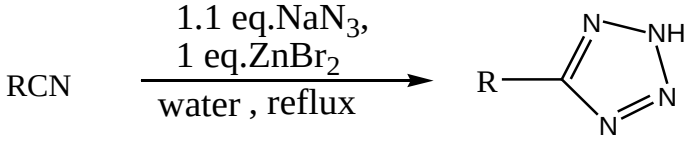


Hydrolysis, followed by decarboxylation, produced a compound having the formula **$C_7H_6N_4$,** the **$C_6H_5CN_4$** unit remaining intact throughout these transformations [24]. During his study of dicyanophenylhydrazine, Bladin had prepared numerous triazoles, and the possibility of forming a nitrogen heterocycle with one more ring nitrogen was a logical extension of his interpretations. The following year Bladin proposed the name ‘‘**tetrazole**” for the new ring structure [25] and in **1892** succeeded in preparing tetrazole itself by the following series of reactions [26], starting with the carboxylic acid produced from his phenylcyanotetrazole (Scheme 3.1).

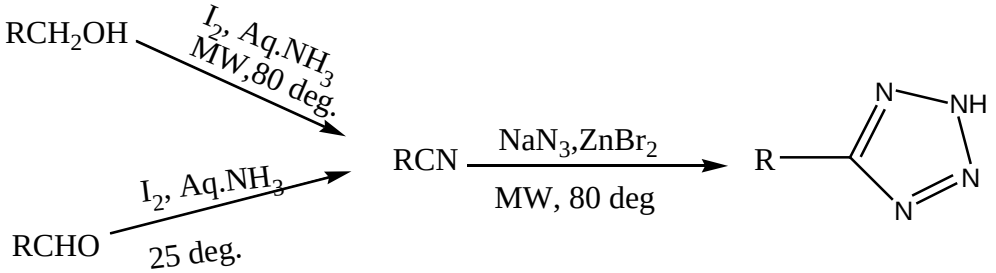


Scheme 3.1: *Synthesis of tetrazole*.

Sharpless and coworkers [27-29] reported synthesis of tetrazoles by refluxing the starting material in water/2-propanol at 80°C with sodium azide and catalytic zinc bromide (Scheme 3.2). The most convenient route to 5-substituted 1*H*-tetrazoles is the addition of azide ion to nitrile [29]. Jim-Min Fang also reported direct transformation of primary alcohols and aldehydes into tetrazoles in aqueous media [30] (Scheme 3.3). The alcohols and aldehydes reacted with iodine in ammonia water to provide the corresponding nitrile intermediates which readily underwent [2 + 3] cycloadditions with sodium azide on exposure to microwave irradiation to give the corresponding triazines and tetrazoles. This method is largely used because ease in workup and high yields. J. Shie and J. Fang, in yet another study have found a direct method for transformation of aldehydes to nitriles by using iodine in ammonia water instead of liquid ammonia or ammonia gas saturated in alcohol solvents [31]. The corresponding nitriles are then cyclized into tetrazoles using NaN_3 and $ZnBr_2$. This transformation utilizes iodine as an appropriate oxidant and presumably proceeds with an intermediate of N-iodo aldimine (Figure 3.2) which eliminates an HI molecule in ammonia solution to afford the nitrile product. This type of tandem reaction in a one-pot procedure provides an expedient route to amides, triazoles and tetrazines besides tetrazoles.

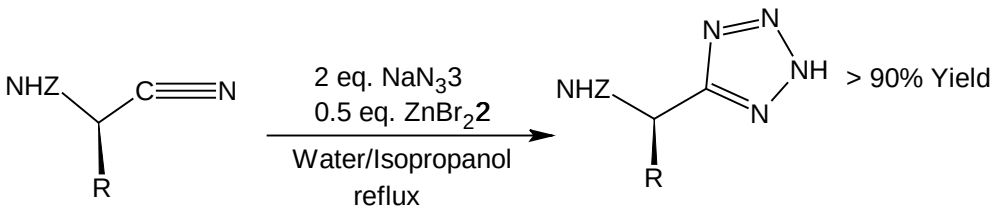


Scheme 3.2



Scheme 3.3: *Direct Conversion of Alcohols and Aldehydes to Tetrazoles*.

Synthesis of α-amino tetrazoles have also been reported by Demko and Sharpless [27] by simple heating of the N-protected α-amino nitrile in a water/2-propanol mixture at reflux (80°C) in the presence of sodium azide and zinc bromide (Scheme 3.4). In another study the same group of workers has reported the synthesis of tetrazoles from nitriles in water using NaN_3 and $ZnBr_2$. The authors have proposed a mechanism of this reaction based on kinetic studies using a water-soluble nitrile [28, 32]. The mechanism of the addition of hydrazoic acid/azide ion to a nitrile to give a tetrazole has been debated, with evidence supporting both a two-step mechanism and a concerted [2 + 3] cycloaddition (Figure 3.3).



Scheme 3.4: *Synthesis of α-Aminotetrazole from an a-Aminonitrile in Water via* Zinc catalysed addition of Azide.

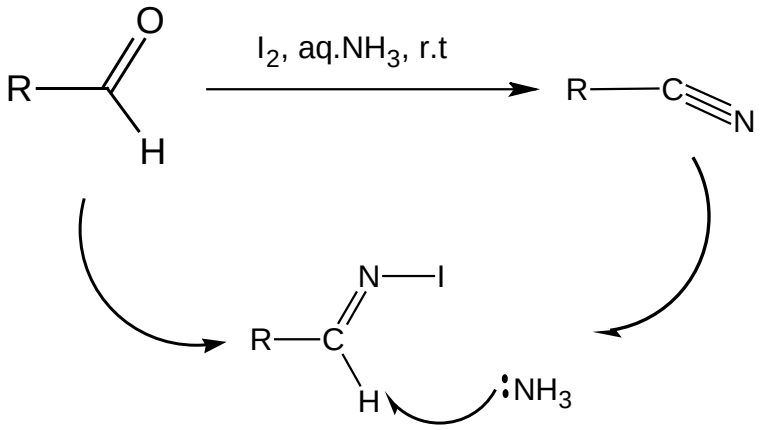


Figure 3.2: *Proposed mechanism of Nitrile formation via intermediate of N-Iodo aldimine.*

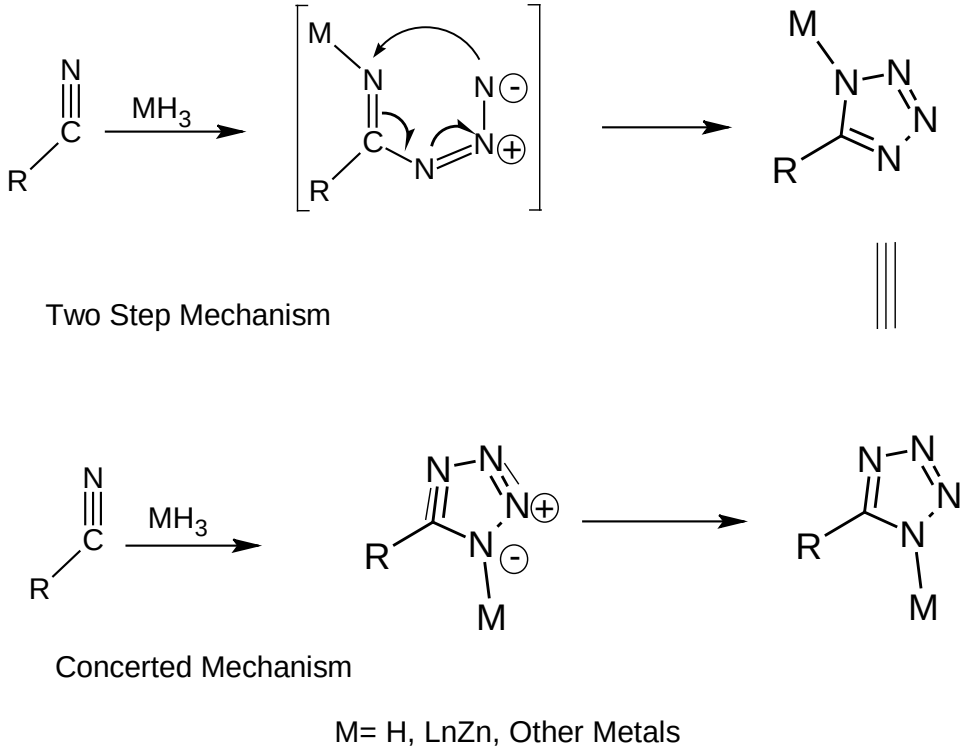
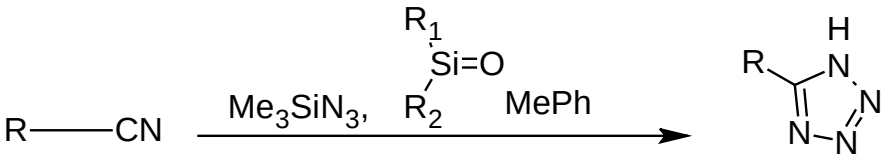


Figure 3.3: *Mechanism of Tetrazole formation.*

A group of workers have reported the synthesis of some unsubstituted tetrazoles from nitriles using trimethylsilyl azide in the presence of dialkylstannyl oxide [33, 34]. A

mechanism for this reaction has been proposed in which trimethylsilyloxydialkyl stannyl azide acts as azidizing agent (Scheme 3.5, Figure 3.4).



Scheme 3.5: *Synthesis of unsubstituted tetrazoles using trialkylazide.*

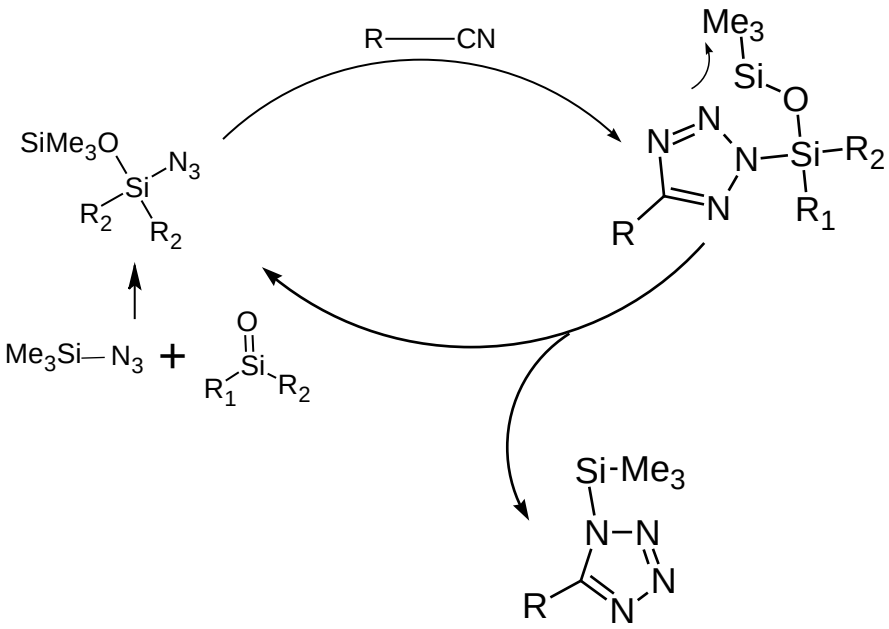
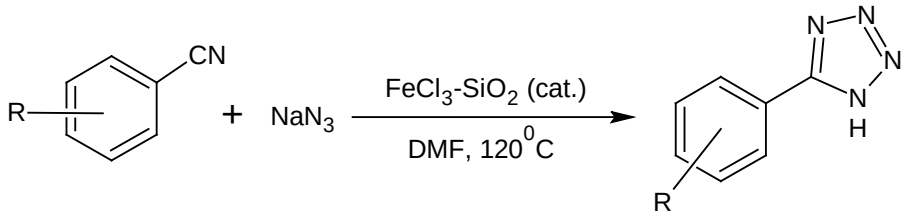


Figure 3.4: *Proposed mechanism of Tetrazole formation using trialkylsilylazide*.

M. Nasrollahzadeh et al., [35] have found an efficient method for the preparation of 5-substituted 1H-tetrazole derivatives, using $FeCl_3$-SiO_2 as an effective heterogeneous catalyst (Scheme 3.6). This method has the advantages of high yields, simple methodology, and easy work-up. The catalyst can be recovered by simple filtration and reused delivering good yields.



Scheme 3.6: *Synthesis of 5-substituted 1H-tetrazoles using $FeCl_3$-SiO_2 as catalyst.*

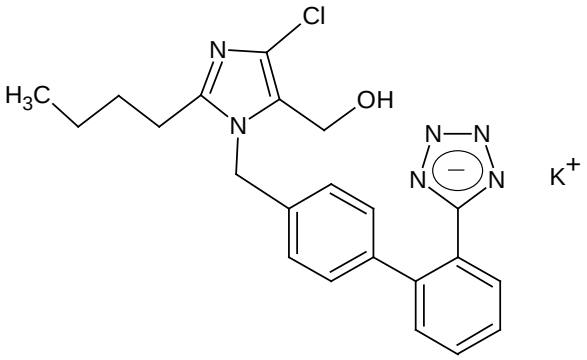
Synthesis of 5-substituted 1*H*-tetrazoles has also been achieved using Tungstates as novel heterogeneous catalysts [36] (Scheme 3.7).

R= different substituents

Scheme 3.7: *Synthesis of 5-substituted 1H-tetrazoles using Tungstates as catalyst.*

A variety of triazole derivatives with a 5-substituted tetrazole structure have revealed strong growth inhibitory activity against *Candida* spp. [37]. Another group of workers have found some 1-(2,4-dihydroxyl thiobenzoyl)-tetrazoles as potential antifungal agents [38]. S.A.F. Rostom et al., have obtained some tetrazole based compounds as potential antimicrobial agents, displaying variable growth inhibitory effects on the tested *Gram positive* and *Gram negative* bacteria with special efficacy against the *Gram positive* strains [39]. Meanwhile, some compounds exhibit moderate antifungal activity against *Candida albicans* and *Aspergillus fumigatus*.

The discovery of a promising non peptide angiotensin receptor antagonist containing a 5-aryltetrazole moiety (Dup 753) **(3.5)** is only one of many examples arising from the impressive amount of work on these derivatives [40, 41].



(3.5)

A **triazine** is one of three organic chemicals, isomeric with each other, whose molecular formula is $C_3H_3N_3$ and whose empirical formula is **CHN**. The triazine structure is a heterocyclic ring, analogous to the six-membered benzene ring but with three carbons replaced by nitrogens. The three isomers of triazine are distinguished from each other by the positions of their nitrogen atoms, and are referred to as 1,2,3-triazine, 1,2,4-triazine, and 1,3,5-triazine (Figure 3.6).

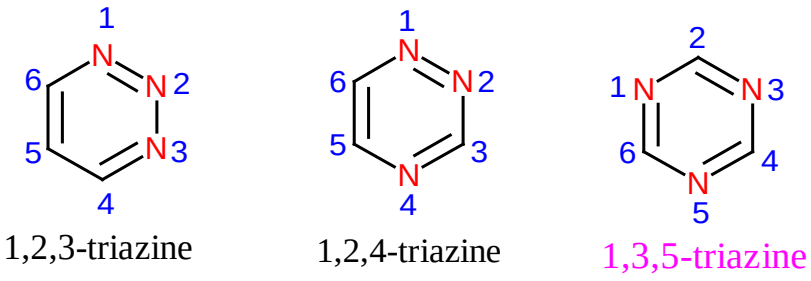
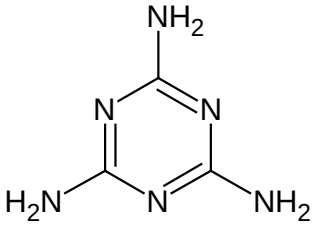


Figure 3.6: *Structural isomers of triazine.*

The best known 1,3,5-triazine derivative is melamine with three amino substituents used in the manufacture of resins **(3.7)**. Another triazine extensively used in resins is benzoguanamine. Triazine compounds are often used as the basis for various herbicides such as cyanuric chloride (2,4,6-trichloro-1,3,5-triazine). Chlorine-substituted triazines are also used as reactive dyes. These compounds react through a

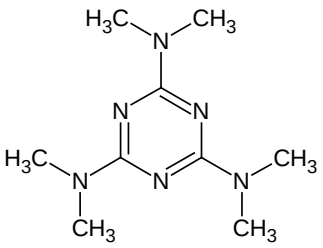
chlorine group with hydroxyl groups present in cellulose fibres in nucleophilic substitution; the other triazine positions contain chromophores. Mixture of Triazines and water are also used to remove H_2S from natural gas.



1,3,5-triazine-2,4,6-triamine

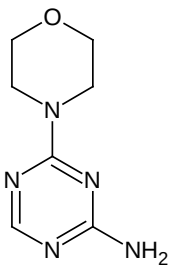
(3.7)

1,3,5-Triazines (or *s*-triazines) are a class of compounds well known for a long time, and still continue the object of considerable interest, mainly due to their applications in different fields, including the production of herbicides and polymer photostabilisers [42]. Some 1,3,5-triazines display important biological properties; for example hexamethylmelamine (HMM, **3.8)** and 2-amino-4-morphlino-*s*-triazine (**3.9**) are used clinically due to their antitumor properties to treat lung breast and ovarian cancer, respectively [43]. Hydroxy methyl penta methylmelamine (HMPMM, **3.10**) is also the hydroxylated metabolite which corresponds to the major active form of HMM [44]. More recently, significant aromatase inhibitory activity were observed for 1,3,5-triazines of general structure **3.11**.

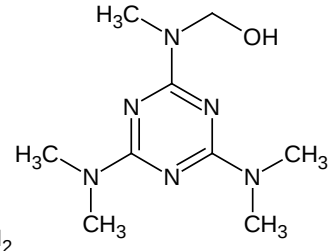


HMM

(3.8)

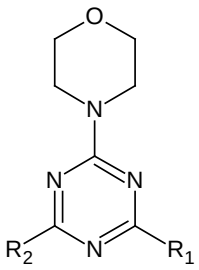


(3.9)



HMPMM

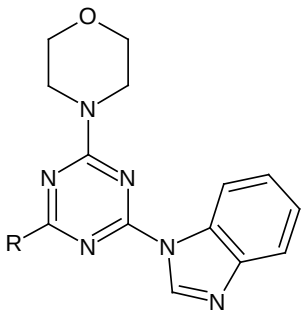
(3.10)



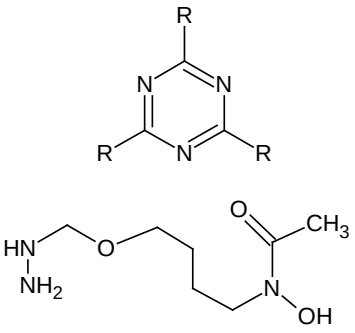
R_1, R_2= Morpholine, Imidazole

(3.11)

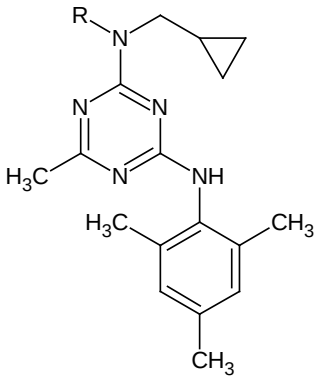
For the similar general structure **(3.12)** antitumor activity in human cancer and murine leukemia cell lines were observed [44]. The 1,3,5-triazine **(3.13)** presents potential use as siderophore (microbial iron shelter) mediated drug [45] and the general structure **(3.14)** presents potent corticotrophin-releasing factor1 receptor antagonist activity [46].



(3.12)

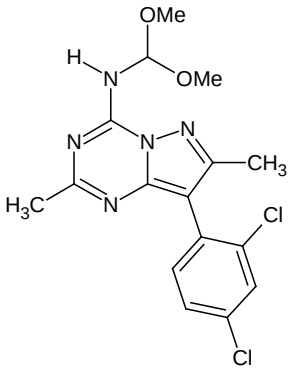


(3.13)

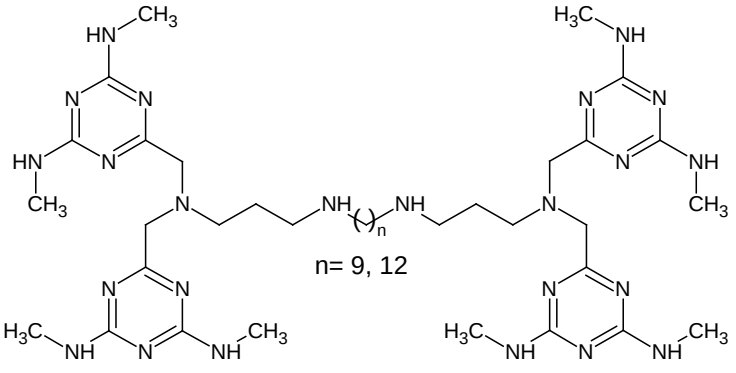


(3.14)

Recently it was discovered that the compound (**3.15)** is a potent corticotrophin-releasing factor1 receptor antagonist [47]. Among several other 1,3,5-triazine substituted polyamines tested, the substrate **(3.16)** presents a good *in vitro* activity against the protozoan parasite *Trypanosoma brucei*, the causative organism of Human African Trypanosomasis [48].

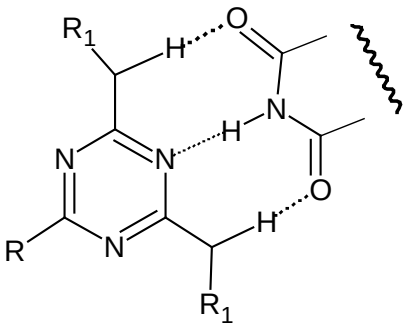


(3.15)



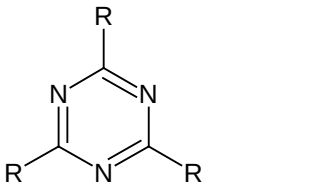
(3.16)

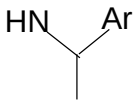
The diverse biological activities observed for different molecules containing the 1,3,5-triazine unit have been further explored in order to discover other new potential molecules through the synthesis of libraries by combinatorial approaches [49]. The 1,3,5-triazine unit has also been used as a key functional group in host-guest chemistry, mainly based on the possibility to generate organized aggregates via the formation of strong three simultaneous hydrogen bonds **(3.17)** [50].



(3.17)

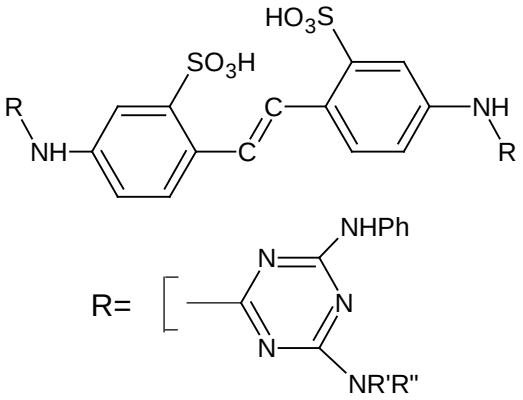
Other applications of the 1,3,5-triazine derivatives are: (i) as chiral stationary phases, for example, the chiral solvating agent **(3.18)** for the determination of enantiomeric excess by NMR spectroscopy [51] and determination of absolute configuration by circular dichroism [52]. (ii) For the preparation of luminescent, optical switches and tri-radical cation species in the case of 2,4,6-triamino-1,3,5-triazine compounds of general structure **(3.19)** [53]. (iii) as metal complexes, liquid crystals, calixarenes, dendrimers, polymers and optical brighteners for household washing powders **(3.20)** [54].





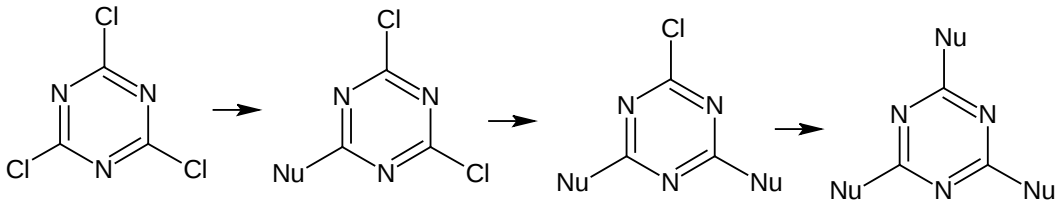
(3.18) R= Cl,

(3.19) R= NAr_2



(3.20)

The most practical method for the synthesis of substituted 1,3,5-triazines is based on the functionalization of the less expensive reagent cyanuric chloride by successive, controlled nucleophilic substitution of each chloride, taking advantage of the decrease of reactivity with the number of substituents [55] (Scheme 3.8). These reactivity profile has been explored in the synthesis of a large number of 1,3,5-triazines containing different substituents, using combinatorial synthesis [49] and for development of solid phase methodologies [56].



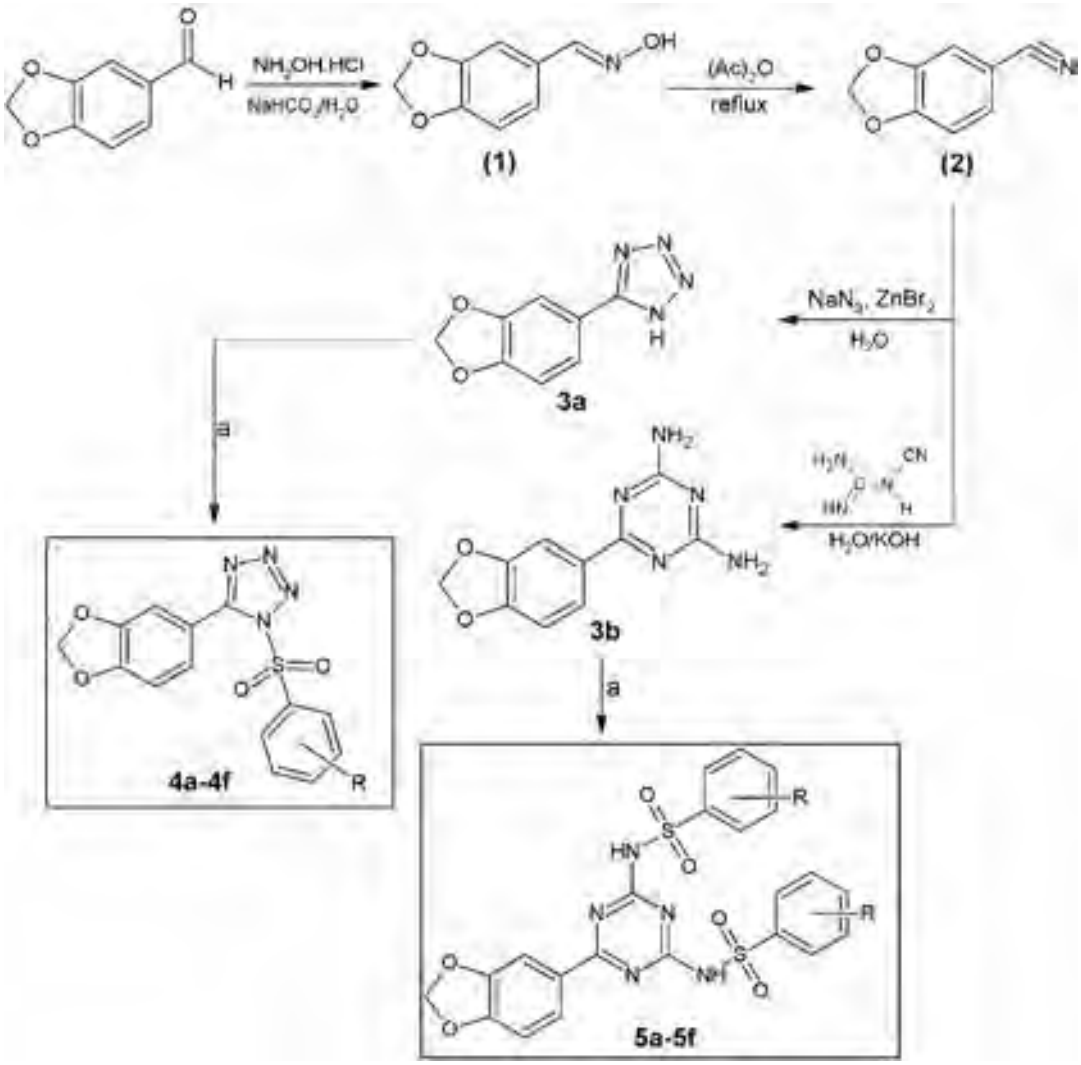
Scheme 3.8: *Ease of chloride substitution on chlorinated 1,3,5-triazines by Nucleophiles (Nu).*

This chapter discusses the synthesis of tetrazole and triazine ring bearing sulfonamide derivatives and their antiamoebic activity.

3.2. RESULTS AND DISCUSSION

3.2.1. Chemistry

Present study was undertaken to synthesize some novel Sulfonamide derivatives of tetrazole and triazine and investigate their probable antiamoebic effects. Target compounds were obtained in a four step reaction procedure as outlined in Scheme 3.9. Piperonal (1,3-benzodioxole-5-carbaldehyde) was converted into 1,3-benzodioxole-5-carbonitrile (**2**) in a two step reaction via an oxime intermediate **(1)**, reported elsewhere [57]. In the third step, the cyclization of the nitrile group of 1,3-benzodioxole-5-carbonitrile **(2)** into 5-(1,3-benzodioxol-5-yl)-1*H*-tetrazole **(3a)** and 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine **(3b)** was achieved by reported method [58, 59]. The target compounds (**4a-4f**) and (**5a-5f)** were obtained in the fourth step by the reaction of 5-(1,3-benzodioxol-5-yl)-1*H*-tetrazole **(3a)** and 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine **(3b)** with different arylsulfonylchlorides (substituted/unsubstituted) respectively, in presence of triethylamine using dry CH_2Cl_2 as a solvent. All the synthesized compounds were characterized by elemental analysis, IR, ^{1}HNMR, ^{13}CNMR and ESI-MS studies and their data are presented in experimental section.



Compound	4a, 5a	4b, 5b	4c, 5c	4d, 5d	4e, 5e	4f, 5f
R	H	4-Me	4-Cl	4-NO_2	2,4-diCl	4-isopropyl

Scheme 3.9: *Synthesis of substituted tetrazole and triazine sulfonamide derivatives*. Reaction conditions: *(a) different sulfonyl chlorides, Et_3N and CH_2Cl_2.*

3.2.2. *In vitro* antiamoebic activity

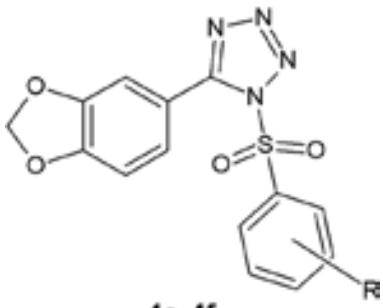
Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds **(4a-4f)** and **(5a-5f)** by microdilution method using HM1: IMSS strain of *Entamoeba histolytica*. The results are summarized in Table 3.1a and 3.1b. The data is presented in terms of percent growth inhibition relative to

untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with the most widely used antiamoebic medication metronidazole with 50% inhibitory concentration (IC_{50}) of 1.80 µM in our experiments. The target compounds under investigation were synthesized from a simple terpene molecule; piperonal and two different pharmacophore groups were incorporated to study their probable antiamoebic effect. The compounds **4a-4f** contain a tetrazole ring while as in compounds **5a-5f** a triazine ring was incorporated in the place of tetrazole ring. Replacement of the tetrazole ring with a triazine ring resulted in a 4 fold improvement in the activity of the key intermediate **3b** (IC_{50}= 3.54 µM) against *Entamoeba histolytica* as compared to **3a** (IC_{50}= 14.24 µM). It is interesting to mention that none of the tetrazole ring bearing derivatives **4a-4f** showed any significant activity (IC_{50} = 3.75-7.56 µM) against the test organism where as all the triazine ring bearing derivatives **5a-5f** showed moderate to excellent activity (IC_{50} = 1.02-2.85 µM). This significant change in activity of these compounds can be attributed to the presence of the triazine ring skeleton or it may be due to the presence of two sulfonamide moieties linked to triazine ring or due to a conjugated effect of both the pharmacophore groups. The presence of different substituents on phenyl ring of the sulfonamide fragment also has a marked effect on the activity of the compounds. In this study it was observed that the compounds bearing a chloro or nitro group at the *para* position of the phenyl ring were more active than the rest of the compounds screened. The presence of methyl group at *para* position decreased the activity and a further chain extension from methyl **(5b)** (IC_{50}= 2.15 µM) to isopropyl **(5f)** at this position in this series of compounds resulted in a pronounced decrease in activity (IC_{50} = 2.85 µM). The presence of chloro group at the *ortho* and *para* position of the phenyl ring in compound **5e** showed moderate activity

similar to the unsubstituted phenyl ring containing compound **5a**. This effect might be due to their drastically conformational change and steric requirement at the *ortho* and *para* position of the phenyl ring of the sulfonamide fragment. These findings indicate that the presence of electron withdrawing groups like chloro and nitro group at the *para* position of the phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds, generally increase the antiamoebic activity of the compounds under study than the compounds bearing electron releasing groups. Tetrazole ring bearing derivatives **(4a-4f)** also adopted a similar behavior but not that significant. The poor antiamoebic activity of the key intermediates and the enhancement of activity with the introduction of $SO_2N<$ group suggest that the sulfonamide moiety was important to activity. Based upon the results it will also be necessary to optimize the led compound by substitution in C-4 position of phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds by more polar groups, which seem to be very important for antiamoebic effect, besides the position of the substituents seems to be an important factor behind the antiamoebic activity of the tested compounds. From the results it can be inferred that the compound **5c** *(N, N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* IC_{50}= 1.05 µM, and **5d** *(N, N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* IC_{50}= 1.02 µM is a promising amoebicidal with high antiamoebic efficacy and least cytotoxicity on human cell line. The results were also statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significant of the difference between the IC_{50} values of metronidazole and the compound **5c** and **5d** was evaluated by *t*-test. The values of the calculated T were found higher than the Table value of T at 5%

level, thus concluding that the character under study is said to be significantly influenced by the treatment.

Table 3.1a: *In vitro* antiamoebic activity of compounds **(4a-4f)** against HM1:IMSS strain of *Entamoeba histolytica* and toxicity profile.



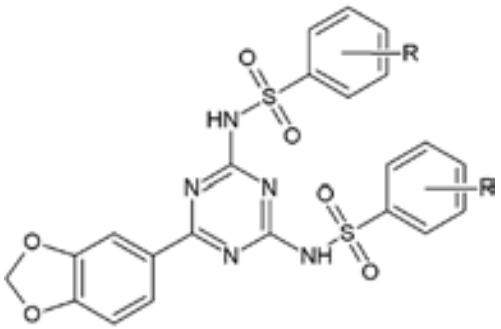
4a-4f

Where R=

Compound	R	Antiamoebic activity		Toxicity Profile	
		IC_{50} (µM)	S.D[a]. (±)	IC_{50} (µM)	Safety Index (SI)
4a	H	7.56	0.14	>100	>13.22
4b	4-Me	6.10	0.20	>100	>16.39
4c	4-Cl	4.85	0.18	>100	>20.61
4d	4-NO_2	3.75	0.23	≈84.7	22.58
4e	2,4-diCl	6.90	0.16	≈85	12.31
4f	4-Isopropyl	6.28	0.14	>100	>15.92
MNZ		1.80	0.20	>100	>55.55

[a]The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation.

Table 3.1b: *In vitro* antiamoebic activity of compounds **(5a-5f)** against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile.



5a-5f

Where R=

Comp.	R	Antiamoebic activity		Toxicity Profile	
		IC_{50} (µM)	S.D[a]. (±)	IC_{50} (µM)	Safety Index(SI)
5a	H	2.60	0.22	>100	>38.46
5b	4-Me	2.15	0.16	>100	>46.51
5c	4-Cl	**1.05**	0.17	>100	>95.23
5d	4-NO_2	**1.02**	0.14	>100	>98.03
5e	2,4-diCl	2.56	0.24	>100	>39.06
5f	4-Isopropyl	2.85	0.17	>100	>35.08
MNZ		1.80	0.20	>100	>55.55

[a]The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation.

3.2.3. *In vitro* cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [60, 61] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [62-64].

In the present study, some newly synthesized compounds were screened for their antiamoebic activity and then evaluated for their cytotoxicity against *Human hepatocellular carcinoma cell line (*HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A sub-confluent population of HepG2 cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13-100 μM. The cell viability (%) obtained with continuous exposure for 48 h are depicted in Figure. 3.21a and 3.21b. The cytotoxicity of all the compounds was found to be concentration-dependent. Figure 1a and 1b depicts that all the compounds including the reference compound metronidazole showed viability ranging from 92.5-100% at the concentration range of 3.13 μM and up to a concentration of 25 μM all the compounds showed a viability of ≥70%. On increasing the concentration range up to 50 and 100 μM the compounds showed moderate to high cytotoxicity against the HepG2 cell line. Except for compounds 4d and 4e all the compounds showed least cytotoxicity and have IC_{50} values greater than 100 μM as given in Table 3.1a and 3.1b. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity

IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Table 3.1a and 3.1b. Compound 5c and 5d showed higher safety index values, better than metronidazole. From the results of antiamoebic activity and cytotoxicity it can be inferred that compound 5c and 5d are least cytotoxic and excellent *Entamoeba histolytica* inhibitors as compared to the reference drug metronidazole (Figure 3.22). These results also showed that the compounds 5c and 5d despite of being highly antiamoebic do not show any marked toxicity on human cell line and have safety index values of ≥ 95 which is better than metronidazole.

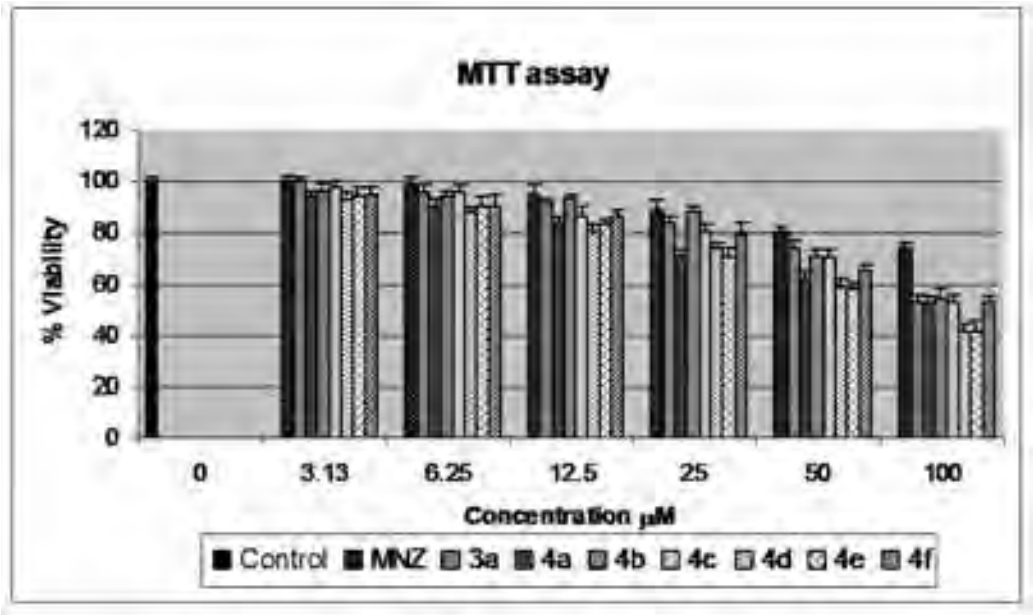


Figure 3.21a: Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with increasing concentration of the compounds.

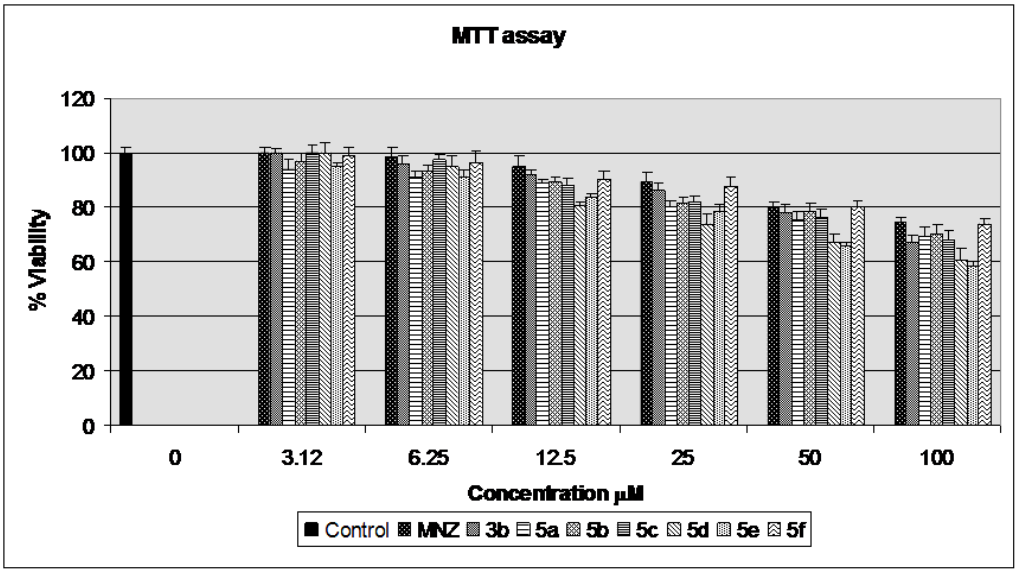


Figure 3.21b: Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with increasing concentration of the compounds.

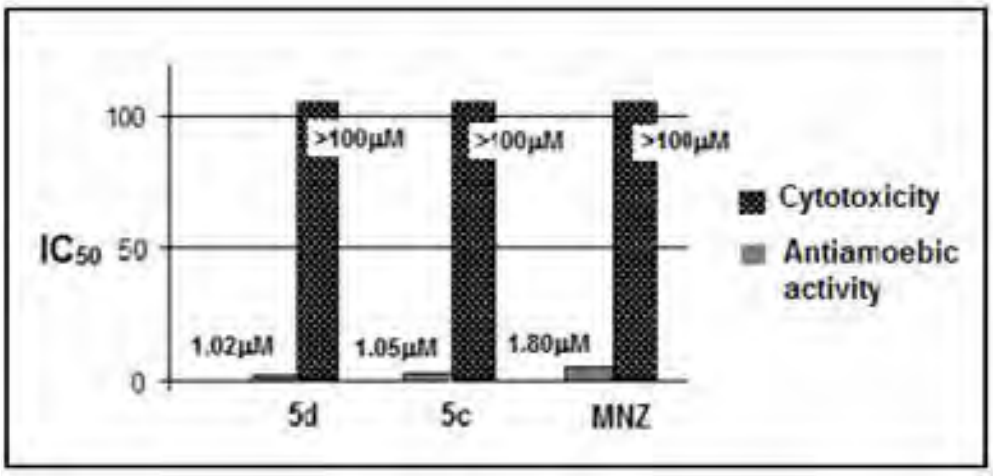


Figure 3.22: Comparison of antiamoebic activity and cytotoxicity profile of compounds 5d, 5c and reference drug metronidazole.

3.3. EXPERIMENTAL

3.3.1. Synthesis

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and are uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ± 0.4% of the theoretical values. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-d_6/$CDCl_3$ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; br s for broad singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapor staining.

3.3.1.1. Synthesis of 5-(1,3-benzodioxol-5-yl)-1H-tetrazole **(3a)**

5-(1,3-benzodioxol-5-yl)-1H-tetrazole was synthesized from 1,3-benzodioxole-5-carbonitrile by a reported method [58]. The required nitrile **(2)** was prepared from Piperonal (1,3-benzodioxole-5-carbaldehyde) *via* oxime formation, followed by dehydration with acetic anhydride, by a reported procedure [57]. Nitrile (2.95 g, 20 mmol), sodium azide (1.43 g, 22 mmol), zinc bromide (4.50 g, 20 mmol), were added to 60 ml of water. 5 mL of isopropanol was also added to stop the formation of

clumps. The reaction mixture was refluxed for 24 h and monitored by TLC. After 24 h HCl (3 N, 30 mL) was added to maintain the PH 1 and ethyl acetate (100 mL) was added. The organic layer was separated and the aqueous layer was extracted with 2×100 mL of ethyl acetate. The combined organic layers were evaporated *in vacuo* and 200 mL of 0.25 N NaOH was added and stirred for 30 min, until the precipitate was dissolved and a suspension of zinc hydroxide was formed. The suspension was filtered, and the solid washed with 20 mL of 1 N NaOH. To the filtrate 50 mL of 3 N HCl was added with vigorous stirring and precipitate was formed. It was filtered and washed with 2 × 50 mL of 3 N HCl and dried in a drying oven to furnish the tetrazole as a white powder.

***(E)-1-(1,3-benzodioxol-5-yl)-N-hydroxymethanimine* (1):** White; Yield 95%; mp. 100-105 ^{o}C; IR ν_{max}cm^{-1}: 3225 (NO-H), 2921 (C-H), 1660 (C=N), 1608, 1500 (C=C, Ar), 937 (N-O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 10.85 (broad s, 1H, N-OH), 7.90 (s, 1H, CH=N-OH), 7.30-6.50 (m, 3H, Ar-H), 5.90 (s, -O-CH$_2$-O-); ^{13}C NMR (DMSO-d_6) d(ppm): 152.3, 148.1, 146.0, 132.6, 123.8, 115.8, 112.6, 101.6 (-O-CH$_2$-O-); ESI-MS m/z: [M^{+}+1] 166.04.

***1,3-benzodioxole-5-carbonitrile* (2):** White; Yield 92%; mp. 95-98 ^{o}C; IR ν_{max}cm^{-1}: 2928 (C-H, Ar), 2204 (CN), 1592 (C=C, Ar); ^{1}H NMR (DMSO-d_6) (ppm): 7.18-6.90 (m, 3H, Ar-H), 6.03 (s, 2H, -O-CH$_2$-O-); ^{13}C NMR (DMSO-d_6) δ (ppm): 153.1, 147.9, 123.1, 123.6, 118.2, 116.1, 113.8, 103.4 (-O-CH$_2$-O-); ESI-MS m/z: [M^{+}+1] 148.02.

***5-(1,3-benzodioxol-5-yl)-1H-tetrazole* (3a):** White solid; Yield 84%; mp. 190-193 ^{o}C; Anal. Calc. for $C_8H_6N_4O_2$: C 50.53, H 3.18, N 29.46%, found: C 50.43, H 3.08, N 29.58%; IR ν_{max}cm^{-1}: 3280 (N-H br stretch), 2864 (C-H, Ar), 1632 (C=N), 1595 (C=C, Ar); ^{1}H NMR (DMSO) δ(ppm): 7.62 (1H, d, *J*= 7.2 Hz), 7.54 (1H, s), 7.16

(1H, d, *J*= 8.1 Hz), 6.15 (2H, s, O-CH_2-O), 4.45 (1H, NH, br s); ^{13}C NMR (DMSO) δ(ppm): 154.8 (C=N), 149.2, 147.6, 121.2, 118.5, 108.2, 106.7, 101.6 (-O-CH_2-O); ESI-MS m/z [M^++1] 191.07.

3.3.1.2. General procedure for the synthesis of 5-(1,3-benzodioxol-5-yl)-1-(phenyl/substituted phenyl sulfonyl)-2H-tetrazoles (4a-4f)

To a solution of 5-(1,3-benzodioxol-5-yl)-1H-tetrazole (1 eq.) and triethylamine (3 eq.) in dry CH_2Cl_2 at 0^{o}C was added aryl sulfonyl chlorides (1.2 eq.). The reaction mixture was stirred at 0^{o}C for about 2 hrs and 4-5 hrs at room temperature. The reaction was monitored by TLC and the reaction mass was quenched with distilled water and extracted with dichloromethane. The combined organic layer was washed with distilled water and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by recrystallization.

5-(1,3-benzodioxol-5-yl)-1-(phenylsulfonyl)-1H-tetrazole **(4a):** Cream solid; Yield 65%; mp. 182-185 ^{o}C; Anal. Calc. for $C_{14}H_{10}N_4O_4S$: C 50.91, H 3.05, N 17.03; found: 50.82, H, 3.01, N, 17.18%; IR ν_{max}cm^{-1}: 3068 (C-H, Ar), 1595 (C=C, Ar), 1690 (C=N), 1174 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.93 (2H, d, *J*= 7.2 Hz) 7.73 (1H, t, *J*= 7.2 Hz), 7.62 (2H, m), 7.05 (1H, dd, *J*= 1.8 Hz; ^{2}*J*= 1.8 Hz), 6.93 (1H, d, *J*= 1.5 Hz), 6.81(1H, d, *J*= 8.1 Hz), 6.04 (2H, s, O-CH_2-O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.8 (C=N), 150.6, 147.3, 137.1, 134.1, 128.9, 123.6, 122.6, 115.7, 113.0, 108.2, (Ar-C), 101.5 (O-CH_2-O); ESI-MS m/z: [M^++1] 331.05.

5-(1,3-benzodioxol-5-yl)-1-[(4-methylphenyl)sulfonyl]-1H-tetrazole **(4b):** Yellow solid; Yield 60%; mp. 220-223 ^{o}C; Anal. Calc. for $C_{15}H_{12}N_4O_4S$: C 52.32, H 3.51, N 16.27; found: C 52.26, H, 3.48, N, 16.35%. IR ν_{max}cm^{-1}: 3068 (C-H, Ar), 1598 (C=C, Ar), 1648 (C=N), 1158 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.90 (2H, d, *J*= 7.8 Hz),

7.65-7.60 (2H, m), 7.02 (1H, dd, *J*= 1.2 Hz; ^{2}J= 1.2 Hz), 6.90 (1H, s), 6.83 (1H, d, *J*= 8.1 Hz), 6.07 (2H, s, O-CH_2-O), 2.83 (3H, s); ^{13}C NMR (DMSO-d_6) δ(ppm): ^{13}C NMR (DMSO-d_6) δ(ppm): 156.4 (C=N), 152.5, 146.3, 143.9, 136.1, 128.6, 127.8, 122.4, 121.6, 116.0, 112.9, 108.9, (Ar-C), 101.5 (O-CH_2-O), 16.4 (CH_3); ESI-MS m/z: [M^++1] 345.06.

5-(1,3-benzodioxol-5-yl)-1-[(4-chlorophenyl)sulfonyl]-1H-tetrazole **(4c):** White solid; Yield 67%; mp. 228-230 ^{o}C; Anal. Calc. for $C_{14}H_9N_4ClO_4S$: C 46.10, H 2.49, N 15.36; found: 46.24, H, 2.38, N, 15.47%. IR ν_{max}cm^{-1}: 3065 (C-H, Ar), 1595 (C=C, Ar), 1645 (C=N), 1328,1147 (S=O), 730 (C-Cl); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.98 (2H, d, *J*= 7.2 Hz), 7.77 (2H, m), 7.08 (1H, dd, *J*= 2.2 Hz; ^{2}J= 2.2 Hz), 6.90 (1H, d, *J*= 2.5 Hz), 6.85 (1H, d, *J*= 8.5 Hz), 6.01 (2H, s, O-CH_2-O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.6 (C=N), 149.8, 147.7, 139.2, 134.1, 128.9, 128.0, 123.6, 122.6, 115.7, 113.0, 108.2, (Ar-C), 101.5 (O-CH_2-O); ESI-MS m/z: [M^++1] 366.00.

5-(1,3-benzodioxol-5-yl)-1-[(4-nitrophenyl)sulfonyl]-1H-tetrazole **(4d):** Yellowish solid; Yield 62%; mp. 210-213 ^{o}C; Anal. Calc. for $C_{14}H_9N_5O_6S$: C 44.80, H 2.42, N 18.66; found: 44.92, H, 2.36, N, 18.74%; IR ν_{max}cm^{-1}: 3068 (C-H, Ar), 1645 (C=N), 1590 (C=C, Ar), 1545, 1350 (NO_2), 1321, 1128 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.13 (2H, d, *J*= 7.8 Hz), 7.76 (2H, m), 7.12 (1H, dd, *J*= 2.8 Hz; ^{2}J= 2.8 Hz), 6.96 (1H, s), 6.93 (1H, d, *J*= 8.7 Hz), 6.07 (2H, s, O-CH_2-O); ^{13}C NMR (DMSO-d_6) δ(ppm): ^{13}C NMR (DMSO-d_6) δ(ppm): 156.4 (C=N), 152.5, 146.3, 143.9, 136.1, 128.6, 127.8, 122.4, 121.6, 116.0, 112.9, 108.9, 107.4 (Ar-C), 101.5 (O-CH_2-O); ESI-MS m/z: [M^++1] 376.03.

5-(1,3-benzodioxol-5-yl)-1-[(2,4-dichlorophenyl)sulfonyl]-1H-tetrazole **(4e):** White solid; Yield 58%; mp. 230-233 ^{o}C; Anal. Calc. for $C_{14}H_8Cl_2N_4O_4S$: C 44.80, H 2.42,

N 18.66; found: 44.68, H 2.47, N 18.73%; IR $\nu_{max}cm^{-1}$: 3062 (C-H Ar), 1590 (C=C, Ar), 1640 (C=N), 1321, 1128 (S=O), 742 (C-Cl), ^{1}H NMR (DMSO-d_6) δ(ppm): 7.89 (1H, d, *J*= 7.5 Hz) 7.58 (1H, s), 7.42 (1H, d, *J*= 7.8 Hz), 7.10 (1H, dd, *J*= 2.5 Hz; ^{2}J= 2.5 Hz), 6.94 (1H, d, *J*= 2.4 Hz), 6.86 (1H, d, *J*= 8.4 Hz), 6.02 (2H, s, O-CH_2-O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.8 (C=N), 148.2, 147.5, 138.4, 136.0, 128.6, 122.6, 120.1, 118.3, 116.4, 112.0, (Ar-C), 101.8 (O-CH_2-O); ESI-MS m/z : [M^++1] 399.94, [M^++2] 400.96.

5-(1,3-benzodioxol-5-yl)-1-[(4-isopropylphenyl)sulfonyl]-1H-tetrazole **(4f):** White solid; Yield 60%; mp. 242-245 ^{o}C; Anal. Calc. for $C_{17}H_{16}N_4O_4S$: C 54.83, H 4.33, N 15.04%; found: 54.76, H 4.10, N 15.26%; IR $\nu_{max}cm^{-1}$: 3032 (C-H, Ar), 1595 (C=C, Ar), 1645 (C=N), 1545, 1350 (NO_2) 1321, 1128 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.95 (2H, d, *J*= 7.8 Hz), 7.54 (2H, d, *J*= 7.4 Hz), 7.11 (1H, dd, *J*= 1.8 Hz; ^{2}J= 1.8 Hz), 6.97 (1H, s), 6.85 (1H, d, *J*= 8.1 Hz), 6.01 (2H, s, O-CH_2-O), 3.83-3.68 (1H, m), 1.89 (6H, s); ^{13}C NMR (DMSO-d_6) δ(ppm): 156.8 (C=N), 152.4, 146.8, 143.5, 135.1, 128.5, 128.0, 126.7, 126.2, 125.2, 120.6, 115.0, (Ar-C), 101.5 (O-CH_2-O), 42.6, 26.5 (Isopropyl); ESI-MS m/z: [M^++1] 373.06.

3.3.1.3. Synthesis of 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine **(3b)**

6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine **(3b)** was synthesized from 1,3-benzodioxole-5-carbonitrile **(2)** by following a reported procedure [59]. The requisite nitrile (2) was prepared from Piperonal (1,3-benzodioxole-5-carbaldehyde), *via* oxime formation, followed by dehydration with acetic anhydride, following a reported procedure [57]. Nitrile (5 m mol), dicyandiamide (5.5 m mol) and KOH (10 m mol) were put in 50 ml water. The reaction mixture was heated at reflux for 12-48 hrs. The

suspended solid products were filtered and rinsed with Et_2O to give a pure diaminotriazine.

6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine **(3b):** Cream solid; Yield: 80%; mp. 220-223 ^oC; Anal. Calc. for $C_{10}H_9N_5O_2$: C 51.95, H 3.92, N 30.29%; found: C 51.82, H 4.12, N 30.37%; IR $\nu_{max}cm^{-1}$: 3408 (NH_2), 2865 (C-H, Ar), 1662 (C=N), 1587 (C=C, Ar); ^1H NMR ($CDCl_3$) δ(ppm): 7.26-6.77 (3H, m), 5.87 (2H, s, O-CH_2-O), 4.70 (4H, NH_2 br s); ^{13}C NMR ($CDCl_3$) δ(ppm): 169.8, 167.9 (C=N), 149.5, 148.6, 125.2, 121.0, 115.8, 113.2 (Ar-C), 102.2 (O-CH_2-O); ESI-MS m/z: [M^++1] 232.07.

3.4.1.4. General procedure for the synthesis of N, N$^/$-6-(1,3-benzodioxol-5yl)-1,3,5-triazine-2,4-diyl di/ bisbenzene/substituted benzene sulfonamides (5a-5f)

To a solution of 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (1.0 eq.) and triethylamine (5.0 eq.) in dry CH_2Cl_2 at 0^{o}C was added aryl sulfonyl chlorides (2.0 eq.). The reaction mixture was stirred at 0^{o}C for about 2 hrs and stirring was continued at room temperature for about 4-5 h (completion of reaction was monitored by TLC). After the completion of reaction the reaction mass was quenched with distilled water and extracted with dichloromethane. Finally the combined organic layer was washed with distilled water again and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by recrystallization.

N, N$^/$-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-dibenzenesulfonamide **(5a):** White solid; Yield 68%; mp. 233-235 ^oC; Anal. Calc. for $C_{22}H_{17}N_5O_6S_2$: C 51.66, H 3.35, N 13.69%; found: C 51.54, H 3.42, N 13.76%; IR $\nu_{max}cm^{-1}$: 3295 (NH), 3025 (C-H, Ar), 1598 (C=C, Ar), 1322, 1147 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 7.68 (2H, NH, br s), 7.52 (2H, d, *J*= 7.8 Hz), 7.36-7.30 (4H, m), 7.02 (1H, d, *J*= 8.1 Hz), 6.91-

6.75 (4H, m), 6.49 (2H, t, *J*= 7.5 Hz), 5.95 (2H, s, O-CH_2-O); ^{13}C NMR ($CDCl_3$) δ(ppm): 174.8, 171.0 (C=N), 150.2, 149.6, 135.7, 134.4, 130.6, 129.8, 126.3, 115.5, 113.6 (Ar-C), 101.2 (O-CH_2-O); ESI-MS m/z: [M^++1] 512.06.

N,N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-methylbenzenesulfonamide **(5b):** Yellow solid; Yield 63%; mp. 230-233 ^oC; Anal. Calc. for $C_{24}H_{21}N_5O_6S_2$: C 53.42, H 3.92, N 12.98%; found: C 53.48, H 3.84, N 13.15%; IR $\nu_{max}cm^{-1}$: 3285 (NH), 3012 (C-H, Ar), 1589 (C=C, Ar), 1329, 1131 (S=O); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.41 (2H, NH, br s), 7.95 (1H, d, *J*= 7.8 Hz), 7.58-7.49 (4H, m), 7.10-7.00 (4H, m), 6.95 (1H, d, *J*= 8.1 Hz), 6.87 (1H, d, *J*= 8.1 Hz), 6.05 (2H, s, O-CH_2-O), 2.69 (6H, s) ; ^{13}C NMR ($CDCl_3$) δ(ppm): 176.4, 172.8 (C=N), 150.6, 149.1, 138.2, 130.2, 128.5, 126.3, 124.7, 115.8, 112.4 (Ar-C), 101.2 (O-CH_2-O), 26.8 (CH_3); ESI-MS m/z: [M^++1] 540.08.

N,N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-chlorobenzenesulfonamide **(5c):** White solid; Yield 67%; mp. 215-218 ^oC; Anal. Calc. for $C_{22}H_{15}N_5Cl_2O_6S_2$: C 45.52, H 2.60, N 12.07; found: C 45.43, H 2.54, N 12.25%; IR $\nu_{max}cm^{-1}$: 3262 (NH), 3052 (C-H Ar), 1594 (C=C, Ar), 1310, 1115 (S=O), 732 (C-Cl); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.96 (2H, NH, br s), 8.15-7.89 (4H, m, Ar-H), 7.45-7.28 (4H, m, Ar-H), 7.17 (1H, d, *J*= 7.8 Hz), 6.72 (1H, d, *J*= 7.8 Hz), 6.43 (1H, d, *J*= 8.5 Hz), 6.02 (2H, s, O-CH_2-O); ^{13}C NMR ($CDCl_3$) δ(ppm): 176.8, 172.0 (C=N), 152.5, 148.6, 137.2, 129.1, 128.2, 125.0, 121.7, 118.0, 116.3 (Ar-C), 101.8 (O-CH_2-O); ESI-MS m/z: [M^++1] 580.98.

N,N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzenesulfonamide **(5d):** White solid; Yield 68%; mp. 205-208 ^oC; Anal. Calc. for $C_{22}H_{15}N_7O_{10}S_2$: C 43.93, H 2.51, N 16.30%; found: C 44.06, H 2.57, N 16.23%; IR $\nu_{max}cm^{-1}$: 3258

(NH), 3042 (C-H, Ar), 1586 (C=C, Ar), 1545, 1350 (NO_2), 1314, 1127 (S=O); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.30 (2H, NH, br s), 7.99-7.82 (4H, m, Ar-H), 7.60-7.48 (4H, m, Ar-H), 7.25 (1H, d, *J*= 7.6 Hz), 6.98 (1H, d, *J*= 8.2 Hz), 6.20 (1H, d, *J*= 8.4 Hz), 6.01 (2H, s, O-CH_2-O); ^{13}C NMR ($CDCl_3$) δ(ppm): 178.2, 176.2 (C=N), 152.5, 151.5, 149.4, 148.6, 145.8, 128.2, 124.8, 121.7, 115.6, 112.0 (Ar-C), 100.8 (O-CH_2-O); ESI-MS m/z: [M^++1] 602.05.

N,N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-(2,4-dichlorobenzene sulfonamide **(5e):** White solid; Yield 60%; mp. 210-213 ^{o}C; Anal. Calc. for $C_{22}H_{13}N_5Cl_4O_6S_2$: C 40.69, H 2.02, N 10.79%; found: C 40.58, H 2.08, N 10.68%; IR ν_{max}cm^{-1}: 3283 (NH), 3015 (C-H, Ar), 1595 (C=C, Ar), 1556, 1343 (NO_2), 1322, 1117 (S=O); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.38 (2H, NH, br s), 7.98-7.83 (2H, m, Ar-H), 7.70-7.58 (2H, m, Ar-H), 7.30 (2H, s), 6.80 (1H, d, *J*= 5.4 Hz), 6.65 (1H, d, *J*= 6.5 Hz), 6.56 (1H, d, *J*= 5.8 Hz), 5.95 (2H, s, O-CH_2-O); ^{13}C NMR ($CDCl_3$) δ(ppm): 176.8, 174.9 (C=N), 149.4, 148.6, 138.8, 137.8, 132.6, 130.6, 128.2, 124.8, 120.7, 115.4, 112.3 (Ar-C), 101.8 (O-CH_2-O); ESI-MS m/z: [M^++1] 647.86, [M^++2] 648.91.

N,N′-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-(4-isopropylbenzene sulfonamide) **(5f):** White solid; Yield 65%; mp. 233-235 ^{o}C; Anal. Calc. for $C_{28}H_{29}N_5Cl_4O_6S_2$: C 56.46, H 4.91, N 11.96%; found: C 56.54, H 5.02, N 11.86%; IR ν_{max}cm^{-1}: 3256 (NH), 3020 (C-H, Ar), 1598 (C=C, Ar), 1533, 1320 (NO_2), 1300, 1125 (S=O); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.80 (2H, NH, br s), 7.85-7.43 (8H, m, Ar-H), 6.95 (1H, d, *J*= 5.8 Hz), 6.55 (1H, d, *J*= 7.5 Hz), 6.20 (1H, d, *J*= 7.2), 5.95 (2H, s, O-CH_2-O), 3.30-3.10 (2H, m), 1.93 (12H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ(ppm): 179.0, 172.6 (C=N), 150.8, 149.8, 148.4, 138.2, 128.2, 128.0, 124.8, 120.7, 115.6, 112.5 (Ar-C), 101.8 (O-CH_2-O), 38.5, 24.0; ESI-MS m/z: [M^++1] 596.18.

3.3.2. *In vitro* antiamoebic assay

All the test compounds **(4a-4f and 5a-5f)** and the key intermediates **3a** and **3b** were screened *in vitro* for antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica* by microdilution method [65]. The detailed procedure of this assay is given in Chapter 2.

3.3.3. Cytotoxicity studies (MTT assay)

3.3.3.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37 ^{0}C in a saturated humidity atmosphere containing 95% air/5% CO_2 [66]. The cell lines were harvested when they reached 80% confluencey to maintain exponential growth.

3.3.3.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [67]. The detailed procedure of this assay is given in Chapter 2.

3.4. CONCLUSION

This study has achieved the efficient synthesis of novel Tetrazole and Triazine ring containing derivatives and examined their *in vitro* antiamoebic and cytotoxic activity. Incorporation of triazine ring in place of tetrazole results in enhancement of antiamoebic activity. These results also clearly document that modification at position-4 of the phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds by some electron withdrawing substituents allows an optimization of these compounds for an effective and probably selective antiamoebic therapy. More importantly, antiamoebic and cytotoxicity studies of these compounds resulted in the fnding of two compounds **5c** *(N, N/-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-chlorobenzene sulfonamide)* IC_{50}= 1.05 µM, and **5d** *(N, N/-6-(1,3-benzodioxol-5yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* IC_{50}= 1.02 µM as stronger *Entamoeba histolytica* inhibitors with least cytotoxicity to Human cells (HepG2) than the standard drug metronidazole IC_{50}= 1.80 µM, which gains some insights into the synthesis or structure modifications of triazine pharmacophore bearing derivatives for the purpose of discovering new antiamoebic drug candidates.

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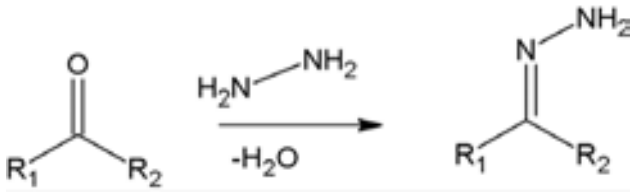
Chapter 4

Synthesis, Characterization and Antiamoebic activity of Acyl-hydrazones and their cyclized 1,3,4-Oxadiazoline analogues

4.1. INTRODUCTION

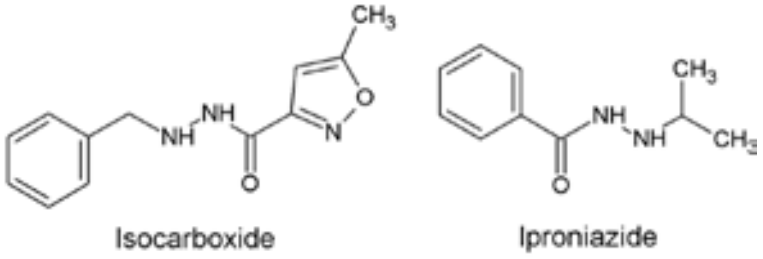
A hydrazone is a class of organic compounds with the structure $R_1R_2C=NNH_2$ [1]. They are related to ketones and aldehydes by the replacement of the oxygen with the NNH_2 functional group. They are formed usually by the action of hydrazine on ketones or aldehydes [2, 3].



Hydrazones are a special group of compounds in the Schiff base family. They are characterized by the presence of >C=N–N=C<. The presence of two inter-linked nitrogen atoms separates them from imines, oximes, etc. Hydrazone Schiff bases of acyl, aroyl and heteroacroyl compounds have an additional donor sites like C=O. As biologically active compounds, hydrazones find applications in the treatment of diseases such as anti-tumor [4], tuberculosis [5], leprosy and mental disorder [6]. Tuberculostatic activity is attributed to the formation of stable chelates with transition metals present in the cell. Thus many vital enzymatic reactions catalyzed by these transition metals cannot take place in the presence of hydrazones [7–9]. Hydrazones also act as herbicides, insecticides, nematocides, rodenticides and plant growth regulators. Hydrazones are used as plasticizers and stabilizers for polymers, polymerization initiators, antioxidants, etc., they act as intermediates in preparative chemistry. In analytical chemistry, hydrazones find application in detection, determination and isolation of compounds containing the carbonyl group. More

recently, they have been extensively used in detection and determination of several metals. They also find applications as indicators and spot test reagents [10].

Many effective compounds, such as isocarboxazide and iproniazide **(4.1)** are synthesized by reduction of hydrazide-hydrazones. Iproniazide, like INH, is used in the treatment of tuberculosis. It has also displays an antidepressant effect and patients appear to have a better mood during the treatment. Another clinicallyPage effective hydrazide-hydrazones is nifuroxazide, which is used as an intestinal antiseptic.



(4.1)

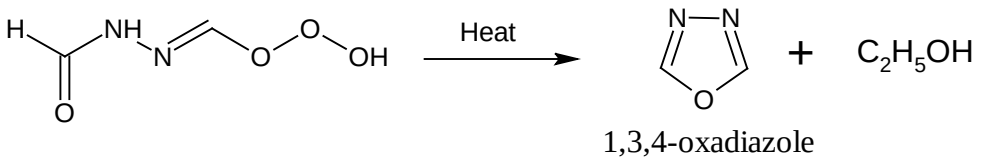
Compounds having a five membered ring containing one oxygen and two nitrogen atoms are called oxadiazoles or in the older literature furadiazoles. [11]. Oxadiazole is considered to be derived from furan by replacement of two methane (-CH=) group by two pyridine type nitrogen (-N=). Four types of oxadiazole are known namely 1,2,3-, 1,2,4-, 1,2,5- and 1,3,4-oxadiazoles (Figure 4.2).

1,2,3-oxadiazoles **1,2,4-oxadiazoles** **1,2,5-oxadiazoles** **1,3,4-oxadiazoles**

(4.2)

Literature survey reveals that particularly 1,3,4-oxadiazole derivatives exhibit wide range of biological activities including anticancer [12], anti-inflammatory [13], fungicidal [14], herbicidal [15], pesticidal, analgesic [16], anticonvulsant [17, 18], anti-HIV [19], antibacterial and plant growth regulator activities [20].

1,3,4-oxadiazole is a liquid, which boils at 150 °C. **Ainsworth** first prepared it in 1965 by the thermolysis of ethylformate formly hydrazone at atmospheric pressure **(Scheme 4.1)**. 1,3,4-oxadiazole is a thermally stable aromatic molecule, other aromatic system are 1,3,4- oxadiazolium cation and the exocyclic-conjugated meso ionic-1,3,4-oxadiazole and 1,3,4- oxadizolines. Also known as derivatives of the non-aromatic reduced system, 2,3 dihydro-1,3,4- oxadiazole, 2,5-dihydro-1,3,4-oxadiozole and 2,3,4,5-tetrahydro-1,3,4-oxadiazole.



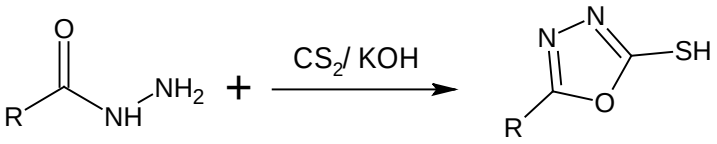
1,3,4-oxadiazole

Scheme 4.1: *Preparation of 1,3,4-oxadiazole.*

Oxadiazole is a very weak base due to the inductive effect of the extra heteroatom [21]. The replacement of two -CH= groups in furan by two pyridine type nitrogen (-N=) reduces aromaticity of resulting oxadiazole ring to such an extent that the oxadiazole ring exhibit character of conjugated diene. The electrophillic substitutions in oxadiazole ring are extremely difficult at the carbon atom because of the relatively low electron density on the carbon atom which can be attributed to electron withdrawal effect of the pyridine type nitrogen atom. However the attack of electrophiles occurs at nitrogen, if oxadiazole ring is substituted with electron-

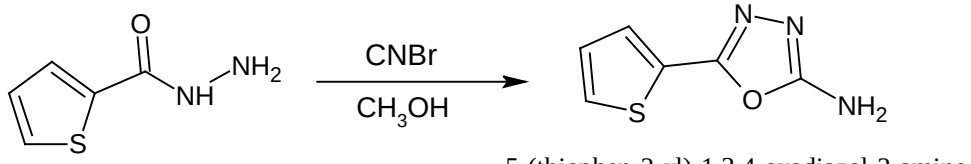
releasing groups. Oxadiazole ring is generally resistant to nucleophilic attack. Halogen-substituted oxadiazole, however, undergo nucleophilic substitution with replacement of halogen atom by nucleophiles. Oxadiazole undergo nucleophilic substitution similarly as occurring at an aliphatic sp^2 carbon atom [22].

M.C Hosur [23] reported synthesis of 2-mercapto-5-aryl-1, 3, 4-oxadiazole **(4.4)** from the properly substituted acid hydrazide **(4.3)** in presence of CS_2/KOH. (Scheme 4.2). This method is very popular since ease in workup and high yields are consistently observed. However, long reaction time is a limiting factor. Number of examples are cited in literature employing this methodology for synthesis of 1, 3, 4-oxadiazole thione/ thiol derivatives [24-27]. M.A. Elborai et al., [28] reported synthesis of 2-amino-5-(2'-thienyl)-1, 3, 4-oxadiazole **(4.6)** by the condensation of 2-thienyl hydrazide **(4.5)** with CNBr (Scheme 4.3). It is a convenient method of synthesis of amino-1, 3, 4-oxadiazole because of shorter reaction time. More reports are cited in literature, which employed this method to obtain the 1, 3, 4- amines [29, 30].



(4.3) **(4.4)**

Scheme 4.2: *Synthesis of 2-mercapto-5-aryl-1, 3, 4-oxadiazole using carbon disulphide in alkaline media.*

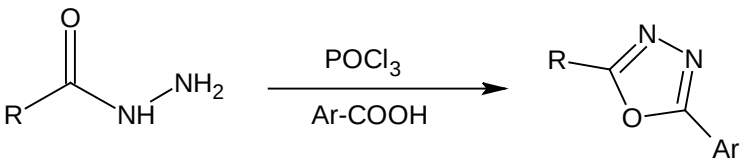


5-(thiophen-2-yl)-1,3,4-oxadiazol-2-amine

(4.5) **(4.6)**

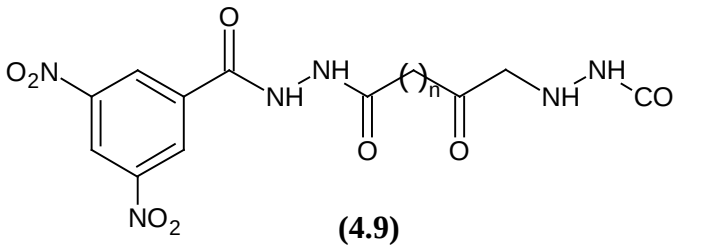
Scheme 4.3: *Synthesis of 1, 3, 4-oxadiazole amine using cyanogen bromide.*

Formation of disubsituted-1,3,4-oxadiazole via cyclodehyrogenation using phosphorus oxychloride ($POCl_3$) has also been accounted in various literatures [31-34]. Condensation of various alkyl hydrazides **(4.7)** with substituted aromatic acids in presence of $POCl_3$ yielded respective 2-alkyl-5-aryl -1, 3, 4-oxadiazoles **(4.8)** (**Scheme 4.4).** N. Sikder et al., [35] synthesized long chain bis-1, 3, 4-oxadiazoles **(4.10)**, from the cyclization of N, N'- dinitrobezoyl malonic or adipic dihydrazide **(4.9)** in presence of $POCl_3$ **(Scheme 4.5)**. Variety of disubstituted 1,3,4-oxadiazoles have been obtained by using $POCl_3$ as dehydrating agent.

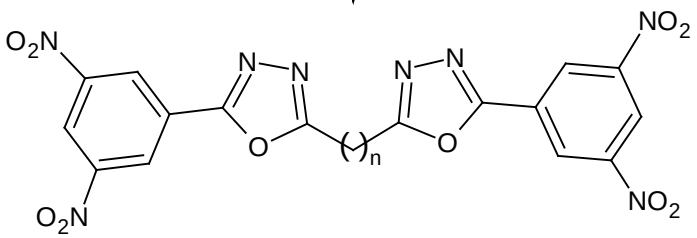


(4.7) **(4.8)**

Scheme 4.4: *Synthesis of 2,5-disubtituted 1,3,4-oxadiazole by cyclodehydrogenation.*



(4.9)

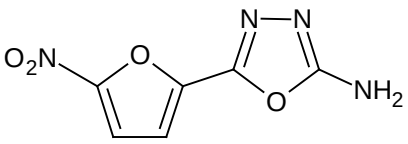


(4.10)

Scheme 4.5: *Synthesis of 1, 3, 4-oxadiazole from semicarbazide*

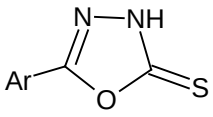
Various oxidizing agents have also been employed to obtain 1,3,4-oxadiazole derivatives from hydrazides, semicarbazides, thiosemicarbazides and Schiff's bases [36, 37].

The capacity of 1,3,4-oxadiazole nucleus to undergo variety of chemical reactions have made it a medicinal backbone on which number of potential molecules can be constructed. A number of researchers have reported antimicrobial activities in 1,3,4-oxadiazoles. A series of 2-amino-5-(5-nitro-2-furyl)-1,3,4-oxadiazoles were screened for their antibacterial activity. In this study, some of the compounds **(4.11)** have shown significant results [38].



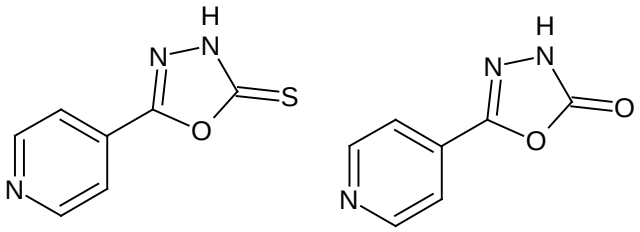
(4.11)

A series of 5-substituted 1,3,4-oxadiazole-2-thiones **(4.12)** have been synthesized for their antibacterial properties [39, 40].



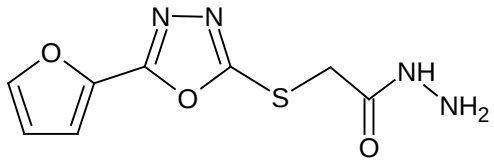
(4.12)

The tuberculostatic and leprostatic properties in a series of 5-(4-pyridyl)-1,3,4-oxadiazole-2 thione, and 5-(4-pyridyl)-1,3,4-oxadiazole-2-one **(4.13)** were reported [41].



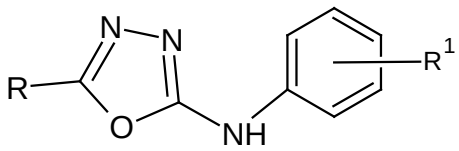
(4.13)

The alpha [5-(2-furyl)-1,3,4-oxadiazol-2-yl-thio] acetohydrazine **(4.14)** showed *in vitro* activity against Mycobacterium tuberculosis [42].



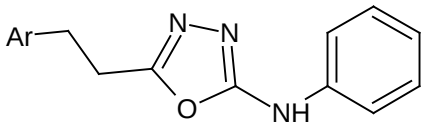
(4.14)

The twelve 2-arylamino-5-aryloxy/arylmethyl-1,3,4-oxadiazoles **(4.15)** derivatives were designed and synthesized for screening against *A. niger* and *H. oryzae* and were found to possess moderate to fairly good antifungal activity [43].



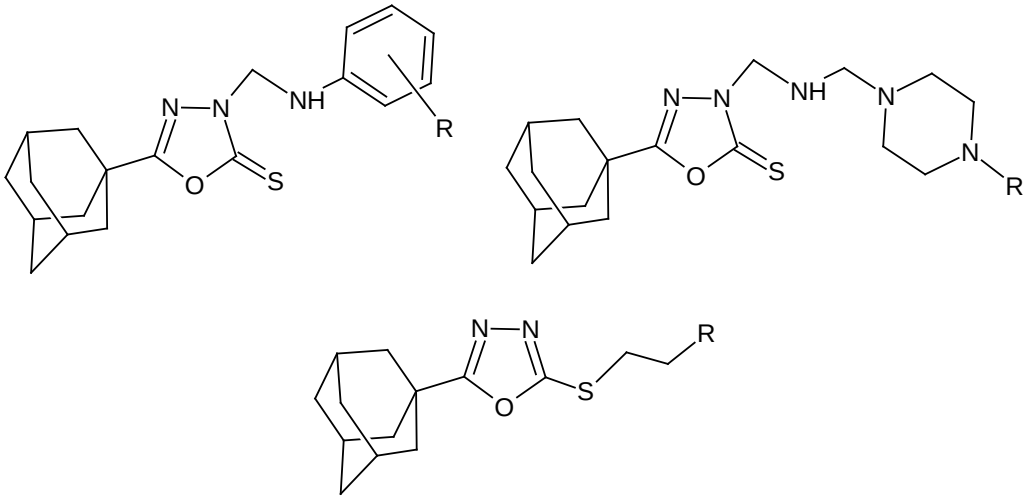
(4.15)

With the aim of the discovering antibacterial compounds, some 2-phenylamino-5-(β-arylethyl)-1,3,4-oxadiazoles **(4.16)** were designed and synthesized and evaluated for antimicrobial properties [44].



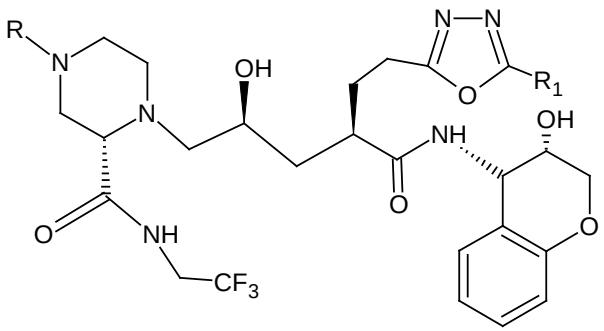
(4.16)

The antimicrobial and anti-HIV-1 activity (using XTT assay) of certain 5-(1-adamatyl)-2-substitutedthio-1,3,4-oxadiazoles and 5-(1-adamantyl)-3-substituted aminomethyl-1,3,4-oxadiazoline-2-thiones has been evaluated **(4.17)** [45].



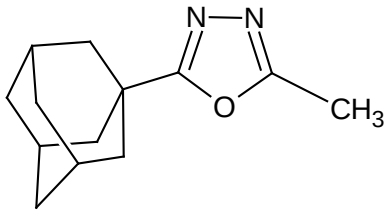
(4.17)

HIV-1 protease inhibitors (PI's) bearing 1,3,4-oxadiazoles at the P1' position **(4.18)** were synthesized by a novel method involving the diastereoselective installtion of a carboxylic acid and conversion to the P1' heterocycle exhibited excellent activities [46].



(4.18)

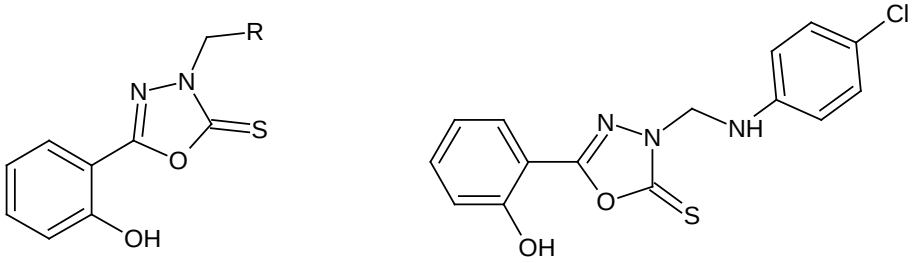
Some novel oxadiazole analogues i.e., 5-(5-methyl-isoxazol-3-yl)-1,3,4-oxadiazoles were synthesized for their antibacterial activities against *E. coli*, *S. aureus* and *P. aeruginosa*. Most of the compounds were found to possess moderate antibacterial activities at 100 ppm concentration using cup-plate agar diffusion method [47]. The antimicrobial and anti-inflammatory activities were reported in novel 2-substituted-5-(1-adamantyl)-1,3,4-oxadiazoles **(4.19)** and 2-substituted-5-(1-adamantyl)-1,3,4-thiadiazoles. Several derivatives showed good or moderate antibacterial activities particularly against the tested Gram-positive bacteria *Bacillus subtilis* and marked antifungal activity against *Candida albicans* [48].



(4.19)

Several other research groups have also reported antimicrobial properties of 1,3,4-oxadiazole derivatives [49-52].

A series of 5-(2-hydroxyphenyl)-3-substituted-2,3-dihydro-1,3,4-oxadiazole-2-thione derivatives **(4.20)** were evaluated for their in-vitro anticancer activity, where seven out of twenty two synthesized compounds displayed high anticancer activity, in the primary assay. These seven oxadiazole compounds were selected for a full anticancer screening against a 60-cell panel assay where they showed non-selective broad spectrum and promising activity against all cancer cell lines. As a result of 60-cell panel assay two oxadiazole compounds were identified as promising lead compounds [53].



(4.20)

Several other research groups have also reported anticancer activities in oxadiazole analogues. [54-56]. Current treatments for chronic hepatitis B virus (HBV) infection include the use of interferon-α and of nucleoside analogs lamivudine, adefovir and entecavir. Besides this recently, anti-hepatitis B virus activities in oxadiazoles has been reported [57]. 1-{2-[5-(1-Benzenesulfonyl-propyl)-[1,3,4]-oxadiazol-2-yl-sulfanyl]-ethyl}-4-(2-meth- oxy-phenyl)-iperazine was found to inhibit the expression of the viral antigens, HBsAg and HBeAg in a concentration-dependent manner with no cytotoxic effects and without any effects on the expression of viral transcripts. The inhibition of virion production was found comparable to that of lamivudine and EC_{50} values of 1.63 and 2.96 μM were obtained for an oxadiazole derivative and lamivudine, respectively.

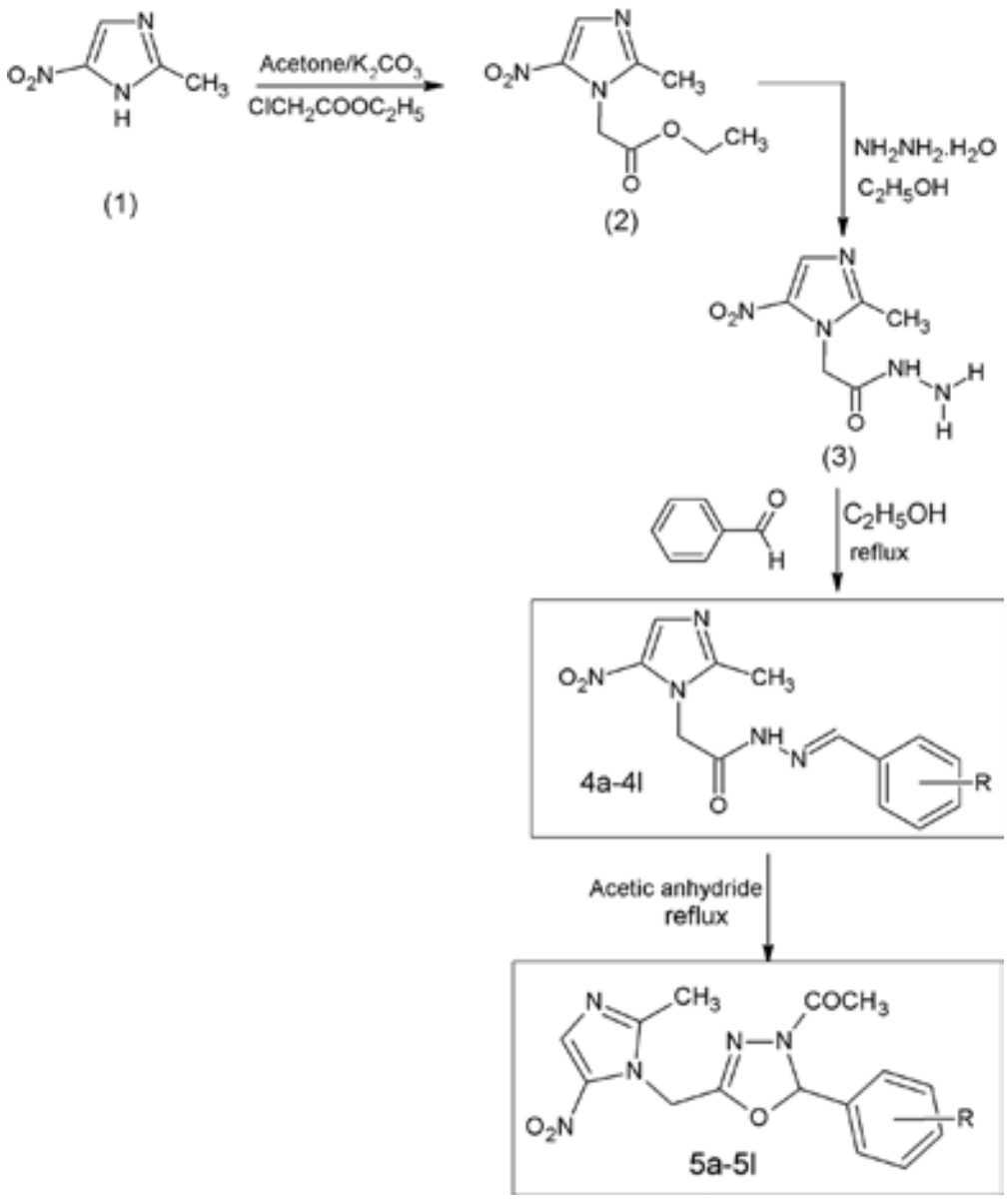
This chapter discusses the synthesis and antiamoebic activity of a series of nitroimidazole based acyl-hydrazones and their cyclized 1,3,4-oxadiazoline analogues.

4.2. RESULTS AND DISCUSSION

4.2.1. Chemistry

Present study was undertaken to synthesize some novel Acyl-hydrazone derivatives and their cyclization into corresponding oxadiazoline derivatives to investigate their probable antiamoebic effects. Target compounds were obtained in a four step reaction procedure as outlined in **Scheme 4.6**. First of all, 2-Methyl-5-nitro-1-imidazo-ethylacetate **(2)** was prepared in dry Acetone from 2-Methyl-5-nitro-1-imidazole **(1)** which was then treated with excess of hydrazine hydrate in ethanol to obtain 2-Methyl-5-nitro-1-imidazo-acethydrazide **(3)**. The Acyl-hydrazone derivatives **(4a-l)** were obtained through a condensation reaction of 2-Methyl-5-nitro-1-imidazo-acethydrazide **(3)** with different aromatic aldehydes in ethanol medium in 1:1 molar ratio. The structure of formed acyl hydrazones was established by elemental analyses and IR, NMR, ESI-MS spectra.

For the synthesis of Oxadiazoline derivatives **(5a-l)** a reported procedure was adapted [58]. The oxadiazoline derivatives were synthesized by refluxing the appropriate hydrazone intermediates in acetic anhydride for 1-5 h under dry conditions **(Scheme 4.6)**. The reaction mixture was poured into ice-water, and the solid product was collected and washed with water. Most of the products were found to be homogeneous by TLC and 300 MHz (300.13 MHz) ^{1}H NMR analyses and heterogeneous products were purified by column chromatography using a methanol/chloroform as eluent. Oxadiazoline product yields ranged from 45-78%. The structure of all the compounds was confirmed using NMR, IR, mass spectrometry, and elemental analysis.



Scheme 4.6: Synthesis of acyl-hydrazones and their cyclized 1,3,4-oxadiazoline analogues. *Where "R" corresponds to different substituents as mentioned in table 4.1 and 4.2 respectively.*

4.2.2. *In vitro* antiamoebic activity

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds (**4a-l**) and (**5a-5l)** by microdilution method using HM1: IMSS strain of *Entamoeba histolytica*. The results are summarized in **Table 4.1** and **4.2.** The data is presented in terms of percent growth inhibition relative to untreated

controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with the most widely used antiamoebic medication metronidazole with 50% inhibitory concentration (IC_{50}) of 1.80 μM in our experiments. The target compounds under investigation were synthesized from a much valuable antiprotozoal nitroimidazole ring. The compounds **4a-4l** contain an acyl-hydrazone linkage which was cyclized and gave the corresponding oxadiazoline products **5a-5l**. The antiamoebic activity of the test compounds seems to be position and substituent dependent. As depicted in **table 4.1** and **4.2** compounds exhibit an interesting inhibition against *HM1:IMSS* strain of *Entamoeba histolytica*. Among the synthesized compounds the series **4a-4l** bearing an acyl-hydrazone moiety and having a free –NH and C=O group showed an interesting inhibitory behavior which may be due to the presence of different substituents on the phenyl ring of the acyl-hydrazone moiety. It is worthy to mention that the cyclization of acyl-hydrazones **(4a-4l)** and their corresponding 1,3,4-oxadiazoline analogues **(5a-5l)** resulted in precipitous decrease in the activity, which gives an indication that the presence of free –NH and C=O group is important for activity **(Figure 4.20)**.

The results of antiamoebic activity showed that the compounds bearing electon withdrawing groups at different positions of the phenyl ring of the acyl-hydrazone pendant showed a decrease activity than the compounds bearing electron donating groups. It is however important to mention that the activity also depends on the strength of the group present at the position-4 of the phenyl ring. Presence of weakly activating groups like methyl **(4e)** showed less activity than the methoxy group **(4i)**, which is strongly activating. A simplified approach to understand this can be attributed to the electronic effects that substituents can exert. Electron donating groups with lone pairs (OMe) on the atoms adjacent to the pi-system activate the

aromatic ring by increasing the electron density on the ring through a resonance donating effect. Methyl group without a lone pair however, exerts an inductive effect through the s-system due to electronegative effects. Similar exciting results were obtained with a further chain extention from methyl **(4e)** to ethyl **(4f**) and methoxy **(4i)** to ethoxy **(4j)**. The stronger electron donating groups decrease the activity which corresponds to earlier reports that electron donating groups increase the electron density which makes the compounds effective against microorganisms and enhances their activity [59]. However high electron density causes more difficult diffusion through the cell membrane and substantial activity loss may occur [60]. Similar results were obtained for compounds bearing electron donating groups (Me or OMe) at 2^{nd} and 5^{th} position of the phenyl ring. Compound **4g** having methyl group at these positions showed moderate activity (IC_{50} = 2.58 µM) comparable to the activity of the compound **4j** (IC_{50} = 2.64 µM), having methoxy group at the same positions. The presence of methoxy group at position 3, 4 and 5 of the phenyl ring in compound **4l** further decrease the activity (IC_{50} = 4.12 µM). Thus for a compound an optimum electron density is inevitable so as to gain a significant activity. The compounds **4b**, **4c** and **4d** having electron withdrawing chloro group at *ortho*, *meta* and *para* position respectively, showed a moderate activity however it was higher than the compound **4a** containing an unsubstituted phenyl ring (IC_{50} =4.28 µM).

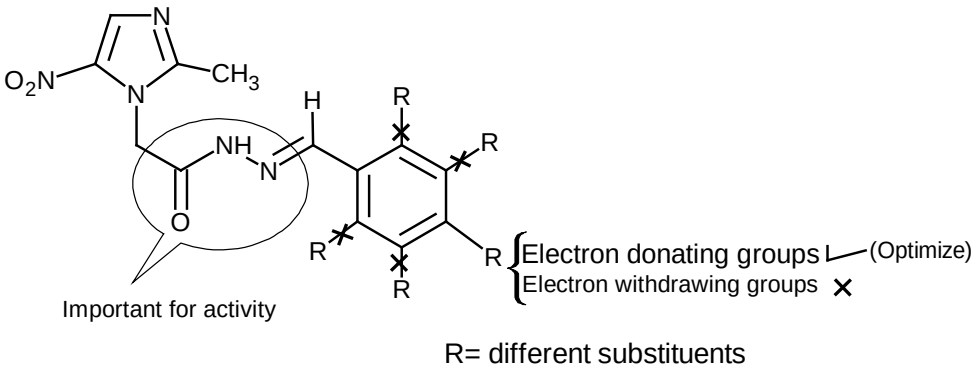
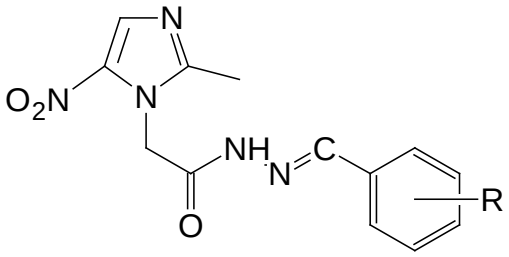


Figure 4.20: Structural requirements for the antiamoebic activity of substituted/ unsubstituted nitroimidazole ring bearing acyl-hydrazones.

As discussed earlier the antiamoebic activity decreased with the cyclization of acyl-hydrazone derivatives **(4a-4l)** to their corresponding oxadiazoline analogues **(5a-5l)** however, the activity was also position and substituent dependent following a similar pattern as that of their parent acyl-hydrazone compounds. Compound **5i** (IC_{50} = 2.18 µM) and **5k** (IC_{50} = 2.32 µM), showed moderate activity; the activity decreased in compounds **5e** (IC_{50} = 2.48 µM) and **5f** (IC_{50} = 2.62 µM). All other compounds showed a further decrease in activity in the range of IC_{50} = 4.38-9.82 µM. Based upon the results it will be necessary to optimize the lead compounds by substitution at C-4 position of the phenyl ring of the hydrazone pendant by electron donating groups, which seems to be very important for antiamoebic activity, besides the position of substituents seems to be very important factor behind the antiamoebic activity of the tested compounds. The results also reveal that the presence of a free –NH and C=O group in the nitroimidazole based acyl-hydrazones is very important for their activity. From the results it can be inferred that the compounds **4e**, **4f**, **4i**, and **4j** showed encouraging results with IC_{50} value in the range of 0.81-1.65 µM, out of which compound **4i** showed the most promising results with 50% inhibitory concentration of

0.81µM. The results were also statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significativity of the difference between the IC_{50} values of metronidazole and the compound **5c** and **5d** was evaluated by *t*-test. The values of the calculated T were found higher than the Table value of T at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

Table 4.1: *In vitro* antiamoebic activity of compounds **(4a-4l)** against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile.



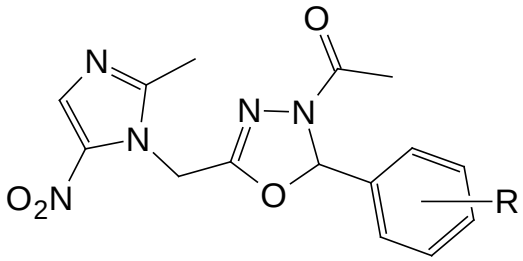
4a-4l

Where R=

Compound	R	Antiamoebic activity		Toxicity Profile	
		IC_{50} (µM)	S.D[a]. (±)	IC_{50} (µM)	Safety Index (SI)
4a	H	4.28	0.28	N.D	N.C
4b	2-Cl	3.86	0.16	N.D	N.C
4c	3-Cl	3.82	0.18	N.D	N.C
4d	4-Cl	3.64	0.22	N.D	N.C
4e	4-Me	**0.96**	0.24	>100	**>104.20**
4f	4-Et	**1.32**	0.22	>100	>75.75
4g	2, 5-DiMe	2.58	0.20	N.D	N.C
4h	4-Isopropyl	3.68	0.24	N.D	N.C
4i	4-OMe	**0.81**	0.18	>100	**>123.45**
4j	4-OEt	**1.65**	0.14	>100	>60.60
4k	2,5-DiOMe	2.64	0.20	N.D	N.C
4l	3,4,5-TriOMe	4.12	0.16	N.D	N.C
MNZ		1.80	0.18	>100	>55.55

[a]The value obtained in at least three separate assays done in triplicate, S.D[a]. (±) Standard deviation.

Table 4.2: *In vitro* antiamoebic activity of compounds **(5a-5l)** against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile.



5a-5l

Where R=

Comp.	R	Antiamoebic activity		Toxicity Profile	
		IC50 (µM)	S.Da. (±)	IC50 (µM)	Safety Index (SI)
5a	H	9.82	0.20	N.D	N.C
5b	2-Cl	6.84	0.18	N.D	N.C
5c	3-Cl	6.32	0.18	N.D	N.C
5d	4-Cl	5.40	0.24	N.D	N.C
5e	4-Me	2.48	0.16	N.D	N.C
5f	4-Et	2.62	0.22	N.D	N.C
5g	2, 5-DiMe	4.38	0.20	N.D	N.C
5h	4-Isopropyl	4.88	0.14	N.D	N.C
5i	4-OMe	2.18	0.18	N.D	N.C
5j	4-OEt	2.32	0.24	N.D	N.C
5k	2,5-DiOMe	4.62	0.22	N.D	N.C
5l	3,4,5-TriOMe	5.82	0.23	N.D	N.C
MNZ		1.80	0.18	>100	>55.55

[a]The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation.

4.2.3. *In vitro* cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [61, 62] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [63-65].

In the present study, some newly synthesized compounds were screened for their antiamoebic activity and the most active compounds were evaluated for their cytotoxicity against *Human hepatocellular carcinoma cell line (*HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A sub-confluent population of HepG2 cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13-100 μM. The cell viability (%) obtained with continuous exposure for 48 h are depicted in **Figure 4.21**. The cytotoxicity of all the compounds was found to be concentration-dependent. **Figure 4.21** depicts that all the compounds including the reference compound metronidazole showed viability ranging from 98-100% at the concentration range of 3.13 μM and up to a concentration of 25 μM all the compounds showed a viability of ≥88%. On increasing the concentration range up to 50 and 100 μM the compounds did not show any remarkable cytotoxicity against the HepG2 cell line. All the tested compounds showed a viability of ≥68% at a concentration of 100 μM as depicted in **Figure 4.21**. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows

estimating the efficacy of compounds. The results are summarized in **Table 4.1** and **4.2.** Compound **4e** and **4i** showed higher safety index values, better than metronidazole. From the results of antiamoebic activity and cytotoxicity it can be inferred that all the tested compounds **4e, 4f, 4i** and **4j** are least cytotoxic and excellent *Entamoeba histolytica* inhibitors as compared to the reference drug metronidazole. These results also showed that the compound **4e** and **4i** despite of being highly antiamoebic do not show any marked toxicity on human cell line and have safety index values of > 104.20 and >123.45 which is better than metronidazole (>55.55).

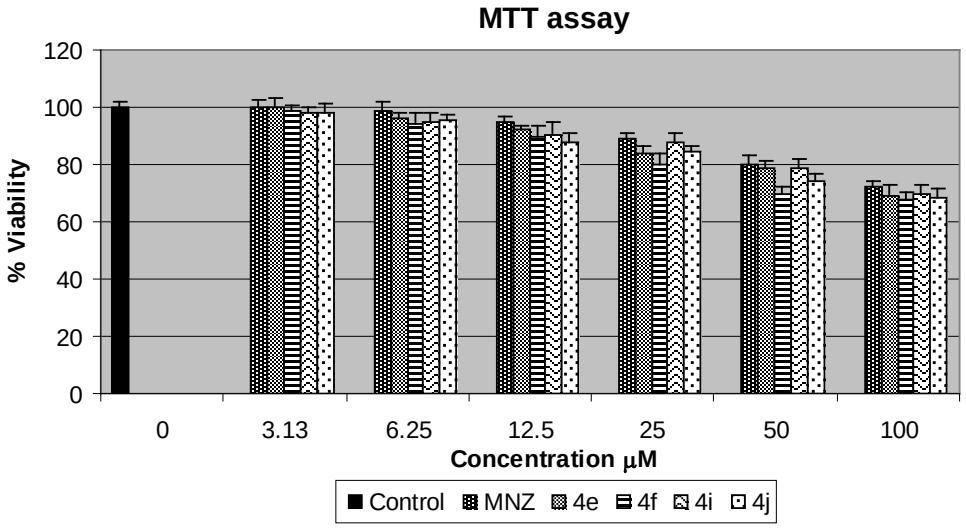


Figure 4.21: Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with Metronidazole, compounds 4e, 4f, 4i and 4j as evaluated by MTT assay.

4.3. EXPERIMENTAL

4.3.1. Synthesis

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp.) were performed using a Mel-temp instrument, and the results were uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ± 0.4% of the theoretical values. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-d6/ $CDCl_3$ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapor staining.

***2-Methyl-5-nitro-1-imidazo-ethylacetate* (2)**

A mixture of 2-methyl-5-nitro-imidazole **1** (1 mmol), ethyl chloroacetate (1 mmol) and potassium carbonate (1.5 m. mol) in dry acetone (5-10mL) was refluxed for 25h. The reaction mixture was filtered hot and the solvent was distilled off from the filtrate. The crude ester thus obtained was purified by recrystallization from ethanol. m.p. 96 ^{0}C; yield 65%.

***2-Methyl-5-nitro-1-imidazo-acethydrazide* (3)**

A mixture of 2-methyl-5-nitro-1-imidazo-ethylacetate **2** (2 mmol) and hydrazine hydrate (2 mmol) in ethanol (10 mL) was refluxed for 10 h. The solution on cooling gave a solid mass of hydrazide **3**, which was collected by filtration, and recrystallized from ethanol. m.p. 189^{o}C; yield 60%.

4.3.1.1. Experimental Procedure for the Synthesis of Acyl-hydrazones (4a-l)

To a stirred solution of hydrazide **3** (1 mmol) and different aromatic aldehydes (1 mmol) in ethanol (25 mL), water (5 mL) was added followed by dropwise addition of glacial acetic acid (0.2 mL). The resulting mixture was refluxed for 5 h, after which the solution was poured into ice water. The mixture was stirred until a precipitate formed, which was collected using suction filtration and dried, followed by recrystallization in aqueous methanol, gave acyl-hydrazones **(4a-l)** in varying yields (70-85%).

***2-(2-methyl-5-nitro-1H-imidazol-1-yl)-N'-[(1E)-phenylmethylene]acetohydrazide* (4a):** White solid; Yield 85%; mp. 198 ^{0}C; Anal. Calc. For $C_{13}H_{13}N_5O_3$: C 54.35, H 4.56, N 24.38 %; found: C 54.40, H 4.67, N 24.29 %; IR $\nu_{max}cm^{-1}$: 3375 (NH), 3078 (C-H stretch), 1665 (C=O), 1621-1465 (C=N and C=C), 1548, 1365 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.76 (1H, br, NH, hydrazone), 8.46 (1H, s, -N=CH-), 7.83 (1H, s, imidazole ring), 7.46-7.10 (5H, m, Ar-H), 4.95 (2H, s, methylenic), 2.39 (3H, s, methyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.9 (C=O), 164.0 (C=N), 145.5, 134.4, 130.5, 129.1, 128.5, 127.0, 125.0, 32.5, 25.5; ESI-MS m/z: [M^{+}+1] 288.10.

***N'-[(1E)-(2-chlorophenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide* (4b):** White solid; Yield 80%; mp. 215-218 ^{0}C; Anal. Calc. For $C_{13}H_{12}N_5O_3Cl$: C 48.53, H 3.76, N 21.77 %; found: C 48.45, H 3.69, N 21.82 %; IR

$\nu_{max}cm^{-1}$: 3358 (NH), 3075 (C-H stretch), 1661 (C=O), 1610-1460 (C=N and C=C), 1550, 1368 ($-NO_2$), 788 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 11.25 (1H, br, NH, hydrazone), 8.52 (1H, s, -N=CH-), 7.80 (1H, s, imidazole ring), 7.55-7.25 (4H, m, Ar-H), 4.83 (2H, s, methylenic), 2.40 (3H, s, methyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=O), 165.0 (C=N), 159.5, 148.0, 135.0, 132.5, 129.5, 128.0, 127.5, 124.5, 31.5, 25.5; ESI-MS m/z: $[M^+ +1]$ 322.08.

***N'-[(1E)-(3-chlorophenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide* (4c):** White solid; Yield 82%; mp. 222-225 ^0C; Anal. Calc. For $C_{13}H_{12}N_5O_3Cl$: C 48.53, H 3.76, N 21.77 %; found: C 48.42, H 3.62, N 21.89 %; IR $\nu_{max}cm^{-1}$: 3362 (NH), 3065 (C-H stretch), 1655 (C=O), 1614-1462 (C=N and C=C), 1548, 1365 ($-NO_2$), 796 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 11.71 (1H, br, NH, hydrazone), 8.35 (1H, s, -N=CH-), 7.75 (1H, s, imidazole ring), 7.40-7.25 (4H, m, Ar-H), 4.78 (2H, s, methylenic) 2.45 (3H, s, methyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.0 (C=O), 165.5 (C=N), 159.0, 145.0, 134.5, 130.0, 129.5, 127.5, 127.0, 126.5, 37.0, 28.0; ESI-MS m/z: $[M^+ +1]$ 322.08.

***N'-[(1E)-(4-chlorophenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide* (4d):** White solid; Yield 72%; mp. 235-238 ^0C; Anal. Calc. For $C_{13}H_{12}N_5O_3Cl$: C 48.55, H 3.75, N 21.77 %; found: C 48.58, H 3.70, N 21.82 %; IR $\nu_{max}cm^{-1}$: 3355 (NH), 3053 (C-H stretch), 1655 (C=O), 1611-1463 (C=N and C=C), 1545, 1370 ($-NO_2$), 779 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 11.37 (1H, br, NH, hydrazone), 8.55 (1H, s, -N=CH-), 7.75 (1H, s, imidazole ring), 7.45-7.21 (4H, m, Ar-H), 4.72 (2H, s, methylenic), 2.35 (3H, s, methyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=O), 166.5 (C=N), 162.5 (C=N), 150.5, 138.0, 135.0, 130.5, 129.0, 127.5, 125.0, 32.0, 28.5; ESI-MS m/z: $[M^+ +1]$ 322.07.

***2-(2-methyl-5-nitro-1H-imidazol-1-yl)-N'-[(1E)-(4-methylphenyl)methylene] acetohydrazide* (4e):** White solid; Yield 70%; mp. 217-220 ^{0}C; Anal. Calc. For $C_{14}H_{15}N_5O_3$: C 55.81, H 5.02, N 23.24 %; found: C 55.78, H 4.94, N 23.16 %; IR ν_{max}cm^{-1}: 3365 (NH), 3060 (C-H stretch), 1658 (C=O), 1609-1458 (C=N and C=C), 1543, 1368 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.50 (1H, br, NH, hydrazone), 8.50 (1H, s, -N=CH-), 7.70 (1H, s, imidazole ring), 7.48-7.29 (4H, m, Ar-H), 4.68 (2H, s, methylenic), 2.35 (3H, s, CH_3 imidazole ring), 2.30 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.0 (C=O), 168.0 (C=N), 164.0, 155.0, 142.5, 138.0, 134.7, 132.9, 129.0, 127.0, 30.5, 25.5, 20.5; ESI-MS m/z: [M^++1] 302.12.

***N'-[(1E)-(4-ethylphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl)aceto hydrazide* (4f):** Cream solid; Yield 84%; mp. 230-233 ^{0}C; Anal. Calc. For $C_{15}H_{17}N_5O_3$: C 57.13, H 5.43, N 22.21 %; found: C 57.24, H 5.35, N 22.35 %; IR ν_{max}cm^{-1}: 3349 (NH), 3050 (C-H stretch), 1662 (C=O), 1611-1463 (C=N and C=C), 1545, 1366 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.65 (1H, br, NH, hydrazone), 8.52 (1H, s, -N=CH-), 7.72 (1H, s, imidazole ring), 7.28-7.15 (4H, m, Ar-H), 4.60 (2H, s, methylenic), 2.80-2.73 (2H, m, CH_2CH_3), 2.30 (3H, s, CH_3 imidazole), 1.98 (3H, t, ethyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 170.5 (C=O), 165.5 ,160.5 (C=N), 155.0, 145.5, 139.0, 135.7, 132.9, 130.0, 129.0, 127.0, 32.5, 20.5, 14.5; ESI-MS m/z: [M^++1] 316.15.

***N'-[(1E)-(2,5-dimethylphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) aceto hydrazide* (4g):** White solid; Yield 70%; mp. 198-200 ^{0}C; Anal. Calc. For $C_{15}H_{17}N_5O_3$: C 57.12, H 5.43, N 22.20 %; found: C 57.05, H 5.26, N 22.09 %; IR ν_{max}cm^{-1}: 3368 (NH), 3057 (C-H stretch), 1668 (C=O), 1609-1455 (C=N and C=C), 1544, 1364 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.40 (1H, br, NH, hydrazone),

8.38 (1H, s, -N=CH-), 7.78 (1H, s, imidazole ring), 7.32 (1H, s, Ar-H), 7.14-7.02 (2H, m, Ar-H), 4.80 (2H, s, methylenic), 2.30 (6H, s, CH_3), 2.00 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.5 (C=O), 165.5 ,160.5 (C=N), 155.0, 145.5, 139.0, 135.7, 132.9, 130.0, 129.0, 127.0, 32.5, 20.5, 14.5; ESI-MS m/z: [M^++1] 316.14.

***N'-[(1E)-(4-isopropylphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide* (4h):** White solid; Yield 80%; mp. 210-213 ^{0}C; Anal. Calc. For $C_{16}H_{19}N_5O_3$: C 58.35, H 5.81, N 21.26 %; found: C 58.37, H 5.77, N 21.25 %; IR ν_{max}cm^{-1}: 3362 (NH), 3060 (C-H stretch), 1665 (C=O), 1618-1453 (C=N and C=C), 1548, 1367 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.70 (1H, br, NH, hydrazone), 8.45 (1H, s, -N=CH-), 7.70 (1H, s, imidazole ring), 7.30-7.18 (4H, m, Ar-H), 4.77 (2H, s, methylenic), 3.25-3.12 (1H, m, -$CH(CH_3)_2$), 2.45 (3H, s, CH_3 imidazole ring), 1.35 (6H, s, -$CH(CH_3)_2$); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.5 (C=O), 165.5, 162.0 (C=N), 150.5, 146.5, 138.0, 135.7, 132.9, 130.0, 129.0, 127.0, 40.5, 35.5, 30.0, 23.5; ESI-MS m/z: [M^++1] 330.15.

***N'-[(1E)-(4-methoxyphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide* (4i):** White solid; Yield 75%; mp. 225 ^{0}C; Anal. Calc. For $C_{14}H_{15}N_5O_4$: C 52.99, H 4.76, N 22.07 %; found: C 53.02, H 4.67, N 22.15 %; IR ν_{max}cm^{-1}: 3370 (NH), 3059 (C-H stretch), 1653 (C=O), 1608-1459 (C=N and C=C), 1543, 1364 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.50 (1H, br, NH, hydrazone), 8.38 (1H, s, -N=CH-), 7.65 (1H, s, imidazole ring), 7.15-6.88 (4H, m, Ar-H), 3.75 (3H, s, OCH_3), 2.42 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.5 (C=O), 166.8, 162.0 (C=N), 148.5, 144.0, 139.5, 134.7, 132.9, 130.0, 129.0, 127.0, 40.5, 55.0, 25.5, 10.5; ESI-MS m/z: [M^++1] 318.12.

N'-[(1E)-(4-ethoxyphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl)aceto hydrazide **(4j):** White solid; Yield 78%; mp. 227-230 ^{0}C; Anal. Calc. For $C_{15}H_{17}N_5O_4$: C 54.38, H 5.17, N 19.32 %; found: C 54.40, H 5.18, N 19.35 %; IR ν_{max}cm^{-1}: 3365 (NH), 3055 (C-H stretch), 1650 (C=O), 1618-1463 (C=N and C=C), 1545, 1360 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.67 (1H, br, NH, hydrazone), 8.42 (1H, s, -N=CH-), 7.68 (1H, s, imidazole ring), 7.15-6.95 (4H, m, Ar-H), 3.95-2.87 (2H, m, OCH_2CH_3), 2.42 (3H, s, CH_3 imidazole ring), 1.35 (3H, t, OCH_2CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.5 (C=O), 166.8, 162.0 (C=N), 148.5, 144.0, 139.5, 134.7, 132.9, 130.0, 129.0, 127.0, 62.5, 24.5, 15.5, 10.5; ESI-MS m/z: [M^++1] 332.14.

N'-[(1E)-(2,5-dimethoxyphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl) acetohydrazide **(4k):** White solid; Yield 80%; mp. 200-203 ^{0}C; Anal. Calc. For $C_{15}H_{17}N_5O_5$: C 51.87, H 4.93, N 20.16 %; found: C 51.76, H 5.02, N 20.08 %; IR ν_{max}cm^{-1}: 3365 (NH), 3068 (C-H stretch), 1658 (C=O), 1610-1460 (C=N and C=C), 1547, 1365 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.50 (1H, br, NH, hydrazone), 8.35 (1H, s, -N=CH-), 7.60 (1H, s, imidazole ring), 7.15 (1H, s, Ar-H), 6.89-6.78 (2H, m, Ar-H), 3.80 (6H, s, OCH_3), 2.46 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 170.5 (C=O), 165.5, 164.0 (C=N), 144.5, 140.0, 138.5, 136.5, 132.5, 130.0, 129.0, 127.0, 58.5, 22.5, 10.5; ESI-MS m/z: [M^++1] 348.13.

2-(2-methyl-5-nitro-1H-imidazol-1-yl)-N'-[(1E)-(3,4,5-trimethoxyphenyl)methylene] acetohydrazide **(4l):** White solid; Yield 72%; mp. 205-207 ^{0}C; Anal. Calc. For $C_{16}H_{19}N_5O_6$: C 50.93, H 5.08, N 18.56 %; found: C 50.95, H 5.05, N 18.60 %; IR ν_{max}cm^{-1}: 3372 (NH) 3068 (C-H stretch), 1660 (C=O), 1612-1456 (C=N and C=C), 1550, 1368 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.63 (1H, br, NH, hydrazone), 8.53 (1H, s, -N=CH-), 7.75 (1H, s, imidazole ring), 6.78 (2H, s, Ar-H), 3.79 (9H, s,

OCH_3), 2.49 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 173.5 (C=O), 164.5, 160.5 (C=N), 153.5, 150.0, 143.5, 141.5, 136.5, 132.5, 130.0, 129.0, 127.0, 110.5, 54.0, 24.5, 12.5; ESI-MS m/z: [M^++1] 378.15.

4.3.1.2. Experimental Procedure for the Synthesis of oxadiazolines (5a-l) via hydrazone intermediates.

All the 1,3,4-oxadiazoline compounds (**5a-l**) were synthesized by addition of acetic anhydride (5 mL) to hydrazones **(4a-l)** (1 mmol), and the resulting solution was refluxed for 1-5 h (Completion of the reaction was monitored by TLC). The reaction mixture was poured into ice water, and the resulting solid product was filtered and washed with copious amounts of water, followed by drying, to give the oxadiazoline compounds **(5a-l)** in varying yields (45-78%); [Hexane/EtOAc (9:1)].

3-acetyl-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2-phenyl-2,3-dihydro-1,3,4-oxadiazole **(5a):** White solid; Yield 45%; mp. 195 ^{0}C; Anal. Calc. For $C_{15}H_{15}N_5O_4$: C 54.71, H 4.59, N 21.27 %; found: C 54.64, H 5.03, N 21.37 %; IR $\nu_{max}cm^{-1}$: 3025 (C-H stretch), 1681 (C=O), 1652 (C=N), 1548, 1359 (-NO_2), 1255 (C-O-C); ^1H NMR (DMSO-d_6) δ(ppm): 7.95 (1H, s, imidazole ring), 7.28-7.15 (5H, m, Ar-H), 6.95 (1H, s, oxadiazole ring), 4.15 (2H, s, methylenic), 2.72 (3H, s, $COCH_3$), 2.45 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=O), 143.0, 140.0, 138.5, 128.6, 125.2, 123.5, 130.0, 89.5, 35.5, 25.0 10.0; ESI-MS m/z: [M^++1] 330.11.

3-acetyl-2-(2-chlorophenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5b):** White solid; Yield 50%; mp. 228-230 ^{0}C; Anal. Calc. For $C_{15}H_{14}N_5O_4Cl$: C 49.53, H 3.88, N 19.25 %; found: C 49.48, H 3.92, N 19.30 %; IR $\nu_{max}cm^{-1}$: 3029 (C-H stretch), 1685 (C=O), 1658 (C=N), 1540, 1353 (-NO_2), 1248 (C-O-C), 793 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 7.89 (1H, s, imidazole ring),

7.20-7.12 (4H, m, Ar-H), 6.58 (1H, s, oxadiazole ring), 4.10 (2H, s, methylenic), 2.62 (3H, s, $COCH_3$), 2.38 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.7 (C=O), 162.3, 160.5, 143.0, 138.5, 130.7, 128.6, 125.2, 123.5, 89.5, 35.5, 21.3, 12.5; ESI-MS m/z: [M^++1] 364.08.

***3-acetyl-2-(3-chlorophenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole* (5c):** White solid; Yield 58%; mp. 215-218 ^{0}C; Anal. Calc. For $C_{15}H_{14}ClN_5O_4$: C 49.54, H 3.88, N 19.25 %; found: C 49.40, H 3.90, N 19.12 %; IR $\nu_{max}cm^{-1}$: 3033 (C-H stretch), 1681 (C=O), 1663 (C=N), 1547, 1352 (-NO_2), 1253 (C-O-C), 789 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 7.83 (1H, s, imidazole ring), 7.18-7.02 (4H, m, Ar-H), 6.73 (1H, s, oxadiazole ring), 4.16 (2H, s, methylenic), 2.58 (3H, s, $COCH_3$), 2.35 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.2 (C=O), 164.8, 161.5, 143.2, 136.8, 132.9, 128.7, 125.0, 123.6, 91.5, 38.0, 25.3, 12.0; ESI-MS m/z: [M^++1] 364.07.

***3-acetyl-2-(4-chlorophenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole* (5d):** Off White solid; Yield 65%; mp. 228-230 ^{0}C; Anal. Calc. For $C_{15}H_{14}ClN_5O_4$: C 49.55, H 3.89, N 19.24 %; found: C 49.50, H 3.93, N 19.09 %; IR $\nu_{max}cm^{-1}$: 3035 (C-H stretch), 1679 (C=O), 1660 (C=N), 1555, 1348 (-NO_2), 1250 (C-O-C), 790 (C-Cl); ^1H NMR (DMSO-d_6) δ(ppm): 7.87 (1H, s, imidazole ring), 7.22-7.07 (4H, m, Ar-H), 6.98 (1H, s, oxadiazole ring), 4.26 (2H, s, methylenic), 2.53 (3H, s, $COCH_3$), 2.37 (3H, s, CH_3 imidazole ring); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.4 (C=O), 162.9, 161.8, 142.8, 137.5, 131.8, 127.4, 124.8, 123.0, 88.9, 36.7, 26.8, 10.0; ESI-MS m/z: [M^++1] 364.07.

***3-acetyl-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2-(4-methylphenyl)-2,3-dihydro-1,3,4-oxadiazole* (5e):** White solid; Yield 78%; mp. 235-237 ^{0}C; Anal. Calc.

For $C_{16}H_{17}N_5O_4$: C 55.97, H 4.99, N 20.40 %; found: C 56.03, H 4.83, N 20.47 %; IR $\nu_{max}cm^{-1}$: 3042 (C-H stretch), 1685 (C=O), 1658 (C=N), 1547, 1352 (-NO_2), 1255 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.86 (1H, s, imidazole ring), 7.15-7.00 (4H, m, Ar-H), 6.95 (1H, s, oxadiazole ring), 4.02 (2H, s, methylenic), 2.49 (3H, s, imidazole ring), 2.37 (3H, s, CH_3 phenyl ring), 2.10 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.8 (C=O), 162.0, 161.3, 142.0, 137.8, 131.0, 127.9, 124.3, 123.8, 90.2, 35.0, 25.3, 10.6; ESI-MS m/z: [M^++1] 344.14.

3-acetyl-2-(4-ethylphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5f):** White solid; Yield 62%; mp. 230-233 ^{0}C; Anal. Calc. For $C_{17}H_{19}N_5O_4$: C 57.14, H 5.36, N 19.60 %; found: C 57.22, H 5.43, N 19.56 %; IR $\nu_{max}cm^{-1}$: 3035 (C-H stretch), 1683 (C=O), 1655 (C=N), 1552, 1349 (-NO_2), 1250 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.79 (1H, s, imidazole ring), 7.26-7.18 (4H, m, Ar-H), 7.02 (1H, s, oxadiazole ring), 4.17 (2H, s, methylenic), 2.98 (2H, q, CH_2CH_3) 2.39 (3H, s, imidazole ring), 2.08 (3H, s, $COCH_3$), 1.98 (3H, s, CH_2CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.9 (C=O), 163.8, 162.5, 142.8, 137.4, 131.6, 127.0, 123.8, 123.0, 87.9, 72.5, 35.0, 25.3, 14.8, 12.0; ESI-MS m/z: [M^++1] 358.15.

3-acetyl-2-(2,5-dimethylphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5g):** Off White solid; Yield 58%; mp. 200-203 ^{0}C; Anal. Calc. For $C_{17}H_{19}N_5O_4$: C 57.14, H 5.36, N 19.60 %; found: C 57.26, H 5.43, N 19.48 %; IR $\nu_{max}cm^{-1}$: 3032 (C-H stretch), 1680 (C=O), 1660 (C=N), 1557, 1348 (-NO_2), 1252 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.83 (1H, s, imidazole ring), 7.32-7.22 (3H, m, Ar-H), 7.06 (1H, s, oxadiazole ring), 4.13 (2H, s, methylenic), 2.43 (3H, s, imidazole ring), 2.35 (6H, s, CH_3 phenyl ring), 2.17 (3H, s, $COCH_3$); ^{13}C NMR

(DMSO-d_6) δ(ppm): 169.4 (C=O), 162.8, 140.7, 139.4, 132.6, 129.7, 124.2, 120.4, 89.3, 76.2, 38.1, 22.6, 10.8; ESI-MS m/z: [M^++1] 358.15.

3-acetyl-2-(4-isopropylphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5h):** Pale yellow solid; Yield 60%; mp. 238-240 ^{0}C; Anal. Calc. For $C_{18}H_{21}N_5O_4$: C 58.21, H 5.70, N 18.86 %; found: C 58.07, H 5.63, N 18.98 %; IR ν_{max}cm^{-1}: 3042 (C-H stretch), 1676 (C=O), 1660 (C=N), 1563, 1352 (-NO_2), 1250 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.92 (1H, s, imidazole ring), 7.26-7.06 (4H, m, Ar-H), 6.96 (1H, s, oxadiazole ring), 4.17 (2H, s, methylenic), 3.30 (1H, s, $CH(CH_3)_2$), 2.48 (3H, s, imidazole ring), 2.27 (3H, s, $COCH_3$), 1.63 (6H, s, $CH(CH_3)_2$); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.3 (C=O), 162.9, 140.1, 138.2, 130.9, 128.2, 124.8, 120.6, 90.5, 74.3, 36.9, 35.0, 25.6, 10.2; ESI-MS m/z: [M^++1] 372.17.

3-acetyl-2-(4-methoxyphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5i):** Cream solid; Yield 45%; mp. 223-225 ^{0}C; Anal. Calc. For $C_{16}H_{17}N_5O_5$: C 53.48, H 4.77, N 19.49 %; found: C 53.29, H 4.80, N 19.32 %; IR ν_{max}cm^{-1}: 3049 (C-H stretch), 1682 (C=O), 1663 (C=N), 1558, 1355 (-NO_2), 1252 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.90 (1H, s, imidazole ring), 7.08-6.89 (4H, m, Ar-H), 6.82 (1H, s, oxadiazole ring), 4.09 (2H, s, methylenic), 3.73 (3H, s, OCH_3), 2.48 (3H, s, imidazole ring), 2.18 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.6 (C=O), 158.7, 151.9, 141.3, 132.7, 128.6, 128.0, 114.1, 89.2, 74.6, 35.8, 35.0,10.5; ESI-MS m/z: [M^++1] 360.13.

3-acetyl-2-(4-ethoxyphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5j):** Cream solid; Yield 68%; mp. 233-235 ^{0}C; Anal. Calc. For $C_{17}H_{19}N_5O_5$: C 54.69, H 5.13, N 19.49 %; found: C 54.65, H 5.18, N 19.50 %; IR ν_{max}cm^{-1}: 3050 (C-H stretch), 1680 (C=O), 1660 (C=N), 1562, 1350 (-NO_2), 1252 (C-

O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.81 (1H, s, imidazole ring), 7.13-6.92 (4H, m, Ar-H), 6.86 (1H, s, oxadiazole ring), 4.10 (2H, s, methylenic), 3.96-3.85 (2H, m, OCH_2CH_3), 2.46 (3H, s, imidazole ring), 2.20 (3H, s, $COCH_3$), 1.55 (3H, t, OCH_2CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.2 (C=O), 155.1, 154.7, 142.3, 130.7, 128.2, 127.3, 112.7, 88.9, 72.7, 65.3, 32.8, 14.8, 10.0; ESI-MS m/z: [M^++1] 374.15.

3-acetyl-2-(2,5-dimethoxyphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3 dihydro-1,3,4-oxadiazole **(5k):** White solid; Yield 70%; mp. 210-214 ^{0}C; Anal. Calc. For $C_{17}H_{19}N_5O_6$: C 52.44, H 4.92, N 17.99 %; found: C 52.38, H 4.87, N 18.03 %; IR $\nu_{max}cm^{-1}$: 3037 (C-H stretch), 1683 (C=O), 1665 (C=N), 1560, 1356 ($-NO_2$), 1253 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.86 (1H, s, imidazole ring), 7.03-6.89 (3H, m, Ar-H), 6.82 (1H, s, oxadiazole ring), 4.08 (2H, s, methylenic), 3.79 (6H, s, OCH_3 phenyl ring), 2.40 (3H, s, imidazole ring), 2.12 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.9 (C=O), 162.4, 157.3, 155.7, 143.6, 128.5, 114.2, 112.6, 89.0, 67.8, 54.6, 37.8, 23.4, 12.6; ESI-MS m/z: [M^++1] 390.14.

3-acetyl-2-(3,4,5-trimethoxyphenyl)-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-2,3-dihydro-1,3,4-oxadiazole **(5l):** Yellow solid; Yield 65%; mp. 205-208 ^{0}C; Anal. Calc. For $C_{18}H_{21}N_5O_7$: C 51.48, H 5.05, N 16.70 %; found: C 51.58, H 4.93, N 16.79 %; IR $\nu_{max}cm^{-1}$: 3029 (C-H stretch), 1676 (C=O), 1661 (C=N), 1557, 1352 ($-NO_2$), 1248 (C-O-C); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.89 (1H, s, imidazole ring), 6.89 (1H, s, oxadiazole ring), 6.25 (2H, s, Ar-H), 4.13 (2H, s, methylenic), 3.80 (9H, s, OCH_3 phenyl ring), 2.48 (3H, s, CH_3 imidazole ring), 2.16 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ(ppm): 169.7 (C=O), 160.8, 156.4, 141.5, 138.9, 137.2, 108.2, 89.7, 73.4, 56.2, 35.8, 23.6, 10.8; ESI-MS m/z: [M^++1] 420.15.

4.3.2. *In vitro* antiamoebic assay

All the compounds **(4a-4l** and **5a-5l)** were screened *in vitro* for antiamoebic activity against *HM1:IMSS* strain of *Entamoeba histolytica* by microdilution method [66]. *Entamoeba histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [67]. The detailed procedure is given in Chapter 2.

4.3.3. Cytotoxicity studies (MTT assay)

4.3.3.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37 ^{0}C in a saturated humidity atmosphere containing 95% air/5% CO_2 [68]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

4.3.3.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [69]. The detailed procedure is given in Chapter 2.

4.4. CONCLUSION

This work lead to the development of nitroimidazole based acylhydrazone derivatives and their cyclized 1,3,4-oxadiazoline analogues as novel *Entamoeba histolytica* inhibitors. Among all the compounds investigated the acylhydrazones were found to be better *Entamoeba histolytica* inhibitors than their corresponding 1,3,4-oxadiazoline analogues. Cyclization resulted in a precipitous decrease in the activity of the compounds, which reveals that the presence of free -NH and C=O group is important for activity, besides the presence and position of substituents played a dominant role on the activity of the compounds, where activity seems to be due to the electron donating and withdrawing nature of the substituents. Compound **4e** and **4i** bearing a free –NH and C=O group and a methyl and methoxy group respectively at *para* position of the phenyl ring happened to be the most potent of all the compounds reported here. Cytotoxicity studies on human hepatocellular carcinoma cell line HepG2 also revealed non cytotoxic nature of the compounds. The most promising results were observed for compound **4i** (*N'-[(1E)-(4-methoxyphenyl)methylene]-2-(2-methyl-5-nitro-1H-imidazol-1-yl)acetohydrazide*) with potent antiamoebic activity (IC_{50} = 0.81 µM), least cytotoxicity (IC_{50} = >100 µM) and safety index value of >123.45, which is better than metronidazole (>55.55).

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Chapter 5

Synthesis, Characterization and Antiamoebic activity of pyrazoline derivatives bearing a tetrazole tail

5.1. INTRODUCTION

One of the important heterocyclic systems which attract progressive interest of many researchers is pyrazoline. Pyrazoline is a dihydro derivative $C_3H_6N_2$ of pyrazole (Figure 5.1). It has only one endocyclic double bond and is basic in nature [1]. Among its various derivatives, 2-pyrazolines seem to be the most frequently studied pyrazoline type compounds [2]. The interest of scientists in such compounds has been stimulated by their biological, pharmacological and industrial importance. For example, 1-unsubstituted-3,5-diaryl-2-pyrazolines were reported to exhibit human acyl CoA cholesterol acyltransferase [3] as well as low-density lipoprotein oxidation inhibitors [4]. Moreover, 1,3,5-triaryl-2-pyrazolines were reported to possess antidepressant properties [5, 6] in addition to monoamine oxidase inhibitory activities [7, 8].

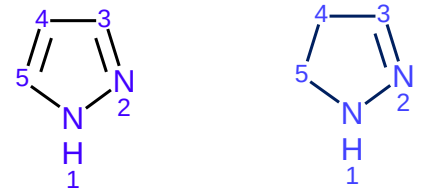
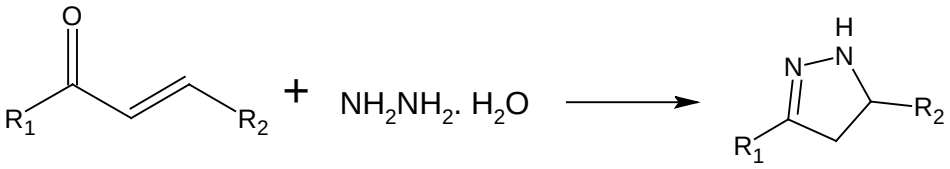


Figure 5.1: *Structure of pyrazole and pyrazoline.*

Pyrazolines are well known and important nitrogen-containing five-membered heterocyclic compounds. Various methods have been worked out for their syntheses [9-12]. The pyrazoline function is quite stable, and has inspired chemists to utilize this stable fragment in bioactive moieties to synthesize new compounds possessing biological activities. Several substituted pyrazolines are found as effective bleaching agents, luminiscents and fluorescents [13].

Pyrazolines have been reported to show a broad spectrum of biological activities including antibacterial, [14-16] antifungal, [17, 18] anti-inflammatory, [19, 20] and anti- depressant activities [21, 22]. Its derivatives, possess a wide range of biological and physiological activities such as anti-implantation, [23] antitumor, [24] antiarthritic, [25] analgesic, [26] immunosuppressive activities [27] and industrial applications. Survey of literature in the recent past reveals that some pyrazoline derivatives also possess cerebroprotective effect [28, 29].

After the pioneering work of Fischer and Knovenagel in the late 19th century, [30] the reaction of α, β-unsaturated aldehydes and ketones with hydrazines (Scheme 5.1) became one of the most popular method for the preparation of 2-pyrazolines [31-36]. As a result, numerous substituted 2-pyrazolines have been synthesized, which has made possible structure- activity relationship investigations of these substances.



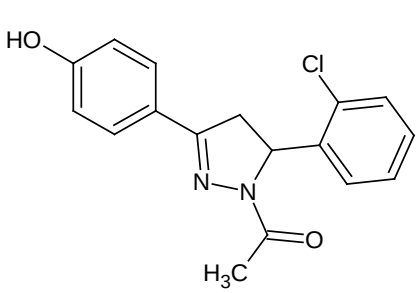
α, β–unsaturated Ketone

Pyrazoline

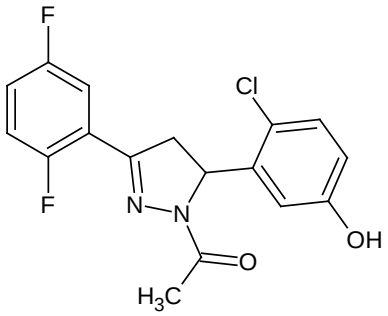
Scheme 5.1: *Synthesis of Pyrazoline*.

Other methods include using ultrasound irradiation and microwave assisted synthesis have been reported with various yields [37]. In recent years solvent free chemical synthesis has developed into a powerful methodology as it reduces the toxic waste produced and therefore becomes less harmful to the environment.

The pyrazoline skeleton is present in a number of pharmacologically active molecules such as phenazone/amidopyrene/ methampyrone (analgesic and antipyretic), azolid/ tandearil (anti-inflammatory), indoxacarb (insecticidal), anturane (uricosuric), etc. Considerable interest has been focused on the pyrazoline structure. The discovery of this class of drugs provides an outstanding case history of modern drug development and also the unpredictability of pharmacological activity from structural modification of a prototype drug molecule and having a variety of medicinal applications. Among the existing various pyrazoline type derivatives, 1-acetyl-pyrazolines have been identified as one of the most promising scaffold in the field of medicinal chemistry. For example *1-[5-(2-chlorophenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl] ethanone* **(5.2)** as a monoamine oxidase inhibitor has proved to be effective in treating Alzheimer's disease, which accounts for most cases of dementia that are diagnosed after the age of 60 years of life [38]. *1-[3-(2,5-diflorophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethanone* **(5.3)** is an inhibiter of kinesin spindle protein (KSP) with potential use for the treatment of cancer [39].



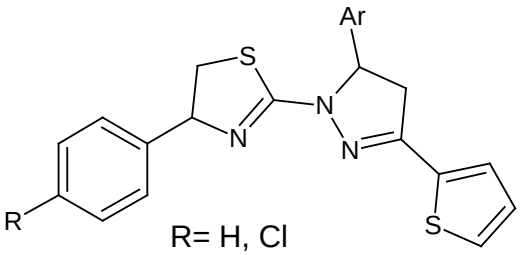
(5.2)



(5.3)

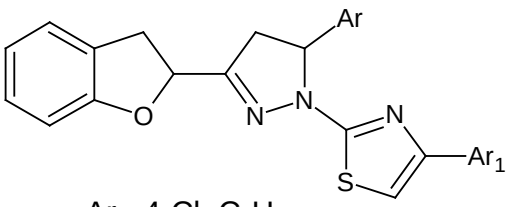
Ozdemir et al., [40] synthesized several 1-(4-Aryl-2-thiazolyl)-3-(2-thienyl)-5-aryl-2-pyrazoline derivatives **(5.4)** and investigated their antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *Streptococcus faecalis*, *Aeromonas hydrophila*, *Candida albicans* and *Candida*

glabrata. A significant level of activity was observed. Abdelwahab et al., synthesized 1-(Benzofuran-2-yl)-4-nitro-3-arylbutan-1-ones and 3-(Benzofuran-2-yl)-4,5-dihydro-5-aryl-1-[4-(aryl)-1,3-thiazol-2-yl]-1H-pyrazoles **(5.5)** and evaluated their antibacterial and antifungal activities at 100 μg concentration [41]. Some of the compounds showed excellent antimicrobial activities than control drugs.



R= H, Cl
Ar= Pyridyl

(5.4)

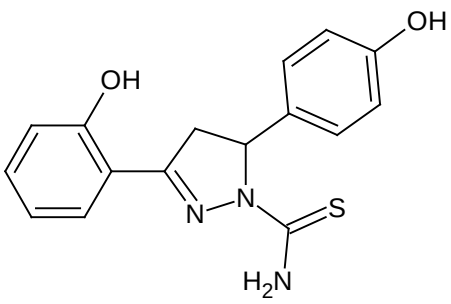


Ar= 4-Cl, C_6H_4
Ar_1= Ph, 4-Br-C_6H_4

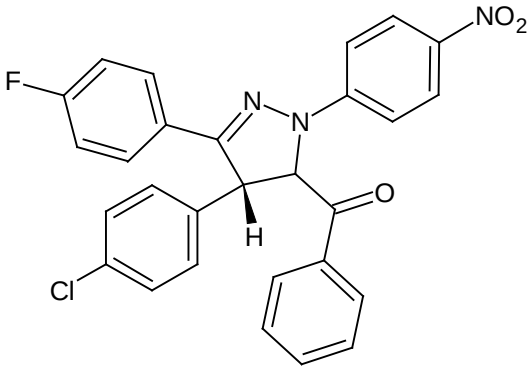
(5.5)

Stirrett et al., [42] synthesized small molecules **(5.6)** with structural similarities to siderophores and evaluated as novel antimicrobials against *Mycobacterium tuberculosis* and *Yersinia pestis*.

Abunada et al., [43] synthesized several 1,3-Diaryl-5-(cyano-aminocarbonyl- and ethoxycarbonyl) -2-pyrazoline, pyrrolo[3, 4-c]pyrazole-4, 6-dione and 1, 3, 4, 5-tetraaryl-2-pyrazoline derivatives **(5.7)** and screened their antimicrobial activities against *E. coli*, *S. aureus*, *Asperagillus flavus* and *C. albicans*.

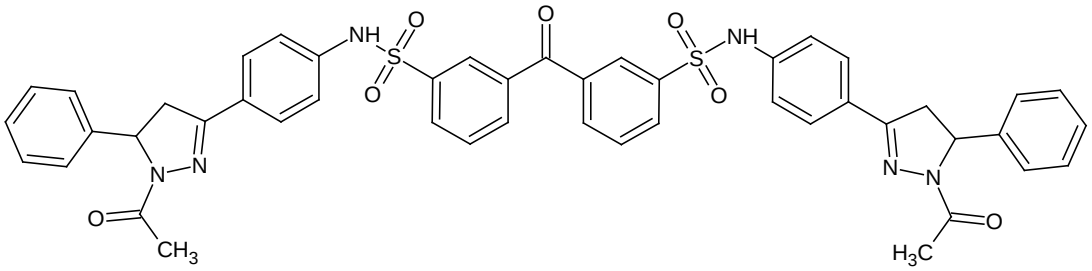


(5.6)

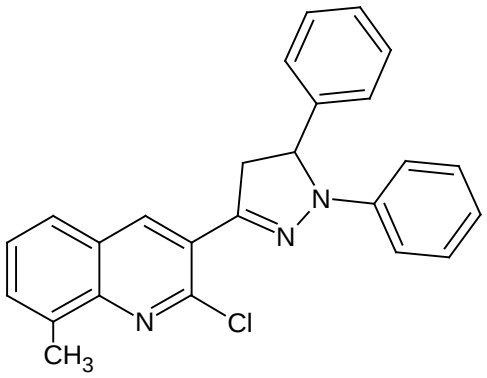


(5.7)

Bhatt et al., [44] synthesized different types of pyrazolines and cyanopyridines **(5.8)** as potential antimicrobial agents. They found that these have remarkable activity against *B. mega*, *B. subtilis*, *E. coli* and *M. tuberculosis* H37 Rv. Bharmal et al., [45] synthesized some pyrazoline derivatives as biologically active agents. All the compounds **(5.9)** showed antimicrobial activity against *S. typhosa* and *A. niger*.

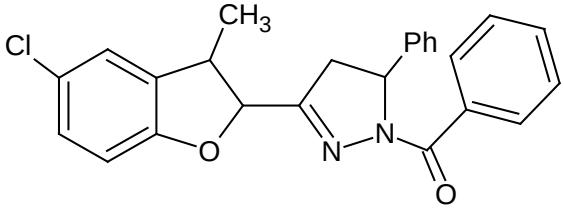


(5.8)



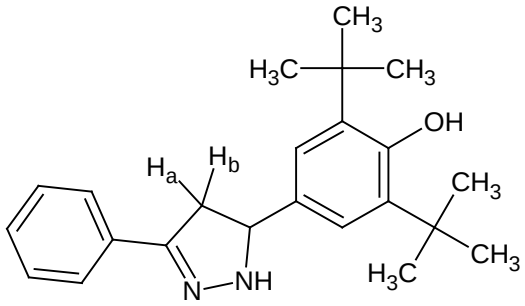
(5.9)

Basawaraj et al., [46] synthesized some 1H-pyrazolines bearing benzofuran **(5.10)** as biologically active agents. They exhibited high antimicrobial activity against *S. aureus* and moderate activity against *E. coli*.



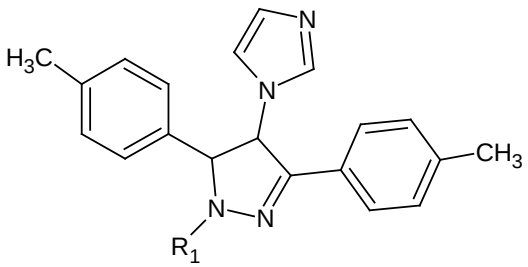
(5.10)

Desai et al., [47] synthesized some new pyrazolines, phenyl pyrazolines, flavanones, and related compounds **(5.11)** and evaluated their antimicrobial activities. The products exhibited activity against Gram +ve bacteria.

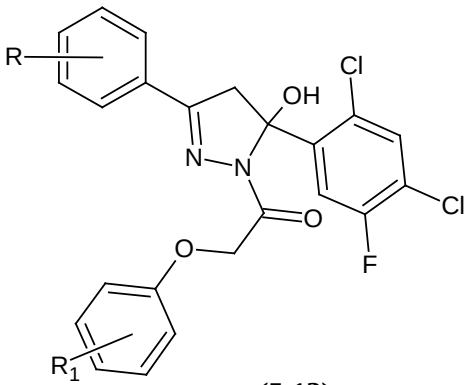


(5.11)

Zampieri et al., reported synthesis of 1-(3,5-diaryl-4,5-dihydro-1H-pyrazol-4-yl)-1H-imidazole derivatives **(5.12)** tested towards a strain of *Candida albicans* and a strain of *Mycobacterium tuberculosis* H37Rv. Imidazole derivatives showed an interesting antifungal and antimycobacterial activity against the tested strains [48]. A series of chlorofluorine containing pyrazoline **(5.13)** were prepared by treatment of chalcone dibromides with aryloxy acid hydrazides in the presence of triethylamine, gave chloro-fluorine containing hydroxy pyrazolines rather than the expected 1-aryloxy-3-aryl-5-aryl pyrazoles. Some compounds showed very good antibacterial and antifungal activity [49].

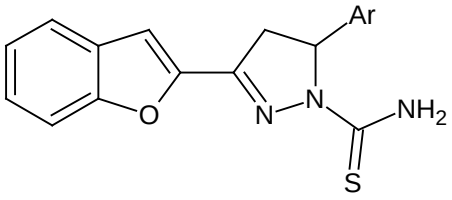


(5.12)

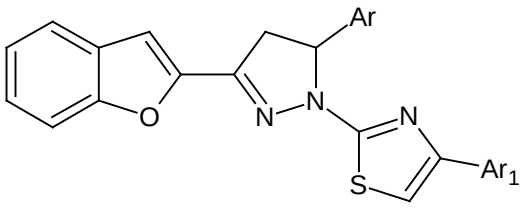


(5.13)

Shaharyar et al., presented a series of N^1-nicotinoyl-3-(4′-hydroxy-3′-methyl phenyl)-5-(substituted phenyl)-2-pyrazolines **(5.14)** and tested in vitro for their antimycobacterial activity. Compounds N^1-nicotinyl-3-(4′-hydroxy-3′-methylphenyl)-5-(1′-chlorophenyl)-2-pyrazoline was found to be the most active agent against MTB and INHR-MTB, with minimum inhibitory concentration of 0.26 μm [50]. Ahmed et al., prepared pyrazolines by the treatment of the Chalcones with nitromethane under Michael addition condition and their subsequent cyclization with thiosemicarbazide under basic refluxing conditions gave 3-(benzofuran-2-yl)-5-(4-aryl)-4,5-dihydropyrazole-1-carbothioamides **(5.15)**. These pyrazolines were further reacted with phenacyl bromides to give thiazole substituted pyrazolines. Some of the compounds showed a significant antimicrobial activity against *Escherichia coli* and *Aspergillus niger* [51].



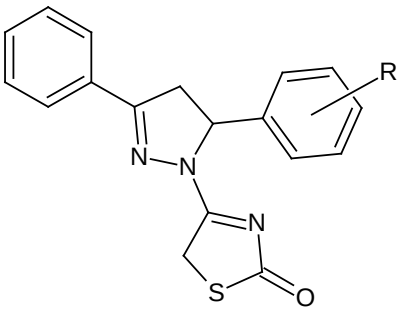
(5.14)



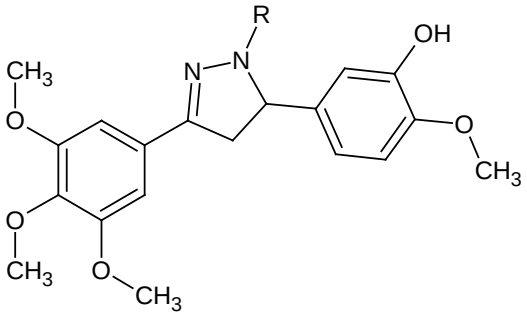
(5.15)

Havrylyuk et al., screened the anticancer activity of several thiazolone-based compounds containing the 5-aryl-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl framework **(5.16)**. The *in vitro* anticancer activity were tested by the National Cancer Institute and most of them displayed anticancer activity on leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancer cell lines and the most efficient anticancer compound was found to be active with selective influence on colon cancer cell lines, especially on HT 29 (log GI50 = - 6.37) [52]. Johnson et al., designed and

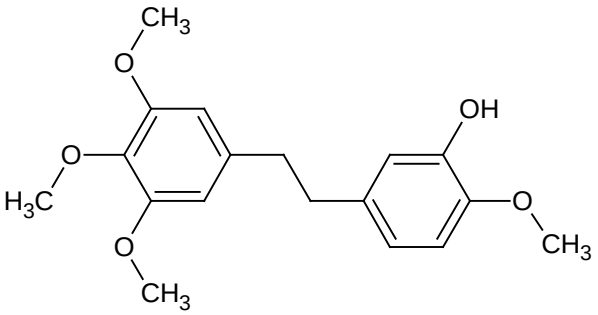
synthesized some new pyrazoline derivatives **(5.17)** which are analogous to combretastatin-A4 **(5.18)** and tested for anticancer activity [53].



(5.16)

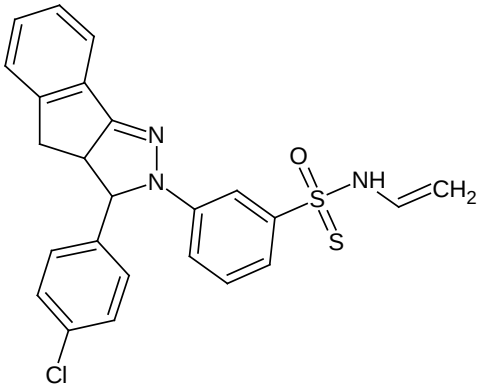


(5.17)

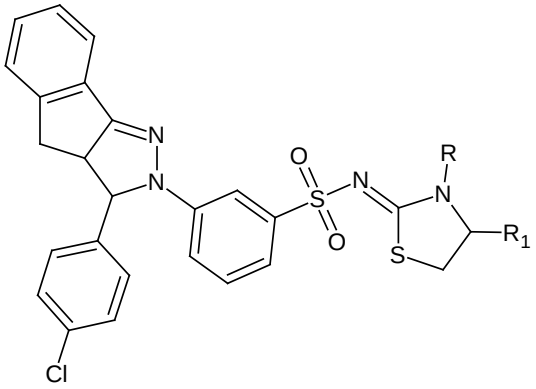


(5.18)

An indene fused series of 3-(4-chlorophenyl)-[1, 2-c]pyrazolines-substituted with benzene sulfonamides, N^1,N^3-disubstituted sulfonylurea and sulfonylthiourea pharmacophores **(5.19, 5.20)** and some derived thiazolidinone and thiazoline ring systems have been synthesized and evaluated for their antitumor activity according to the protocol of the NCI. Eight compounds showed promising broad spectrum antitumor activity against most of the tested sub panel tumor cell lines [54].

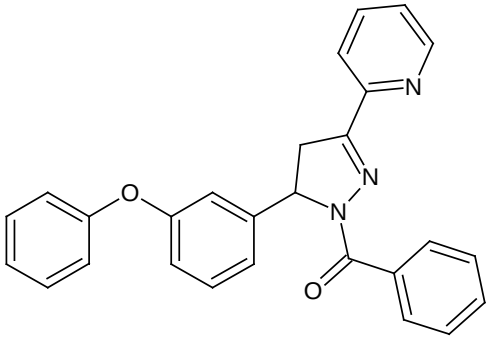


(5.19)

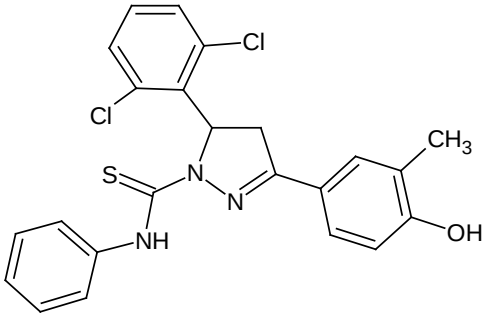


(5.20)

Kini et al., [55] synthesized a novel series of heterocyclic o/m/psubstituted diphenyl ether derivatives **(5.21)** and determined their activity against H37Rv strain of Mycobacterium. All 10 compounds inhibited the growth at concentrations as low as 1μg.ml^{-1}. This level of activity was found comparable to the reference drugs rifampicin and isoniazid at the same concentration. Ali et al., [56] synthesized a series of 5-(-4-(Substituted) phenyl)-3-(4-hydroxy-3-methylphenyl)-4,5-dihydro-1H-1-pyrazolyl-2-toluidino methanethione and 5-(Substituted) phenyl-3-(4-hydroxy-3-methylphenyl)-4,5-dihydro-1H-1-pyrazolyl-2-methoxyanilino methanethione **(5.22)** and tested for their in vitro antitubercular activity against M. tuberculosis H37Rv. Among the synthesized compounds, compound Anilino-3-(4-hydroxy-3-methylphenyl)-5-(2, 6-dichlorophenyl)-4, 5-dihydro-1H-1-pyrazolyl methanethione was found to be more active agent against M. tuberculosis H37Rv with minimum inhibitory concentration of 0.0034 μM.

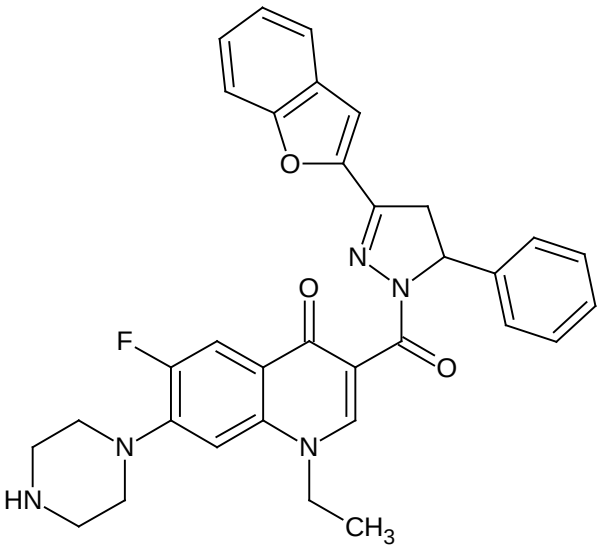


(5.21)



(5.22)

Babu et al., [57] synthesized and evaluated biological activity of 1, 3, 5-Trisubstituted pyrazolines bearing benzofuran **(5.23)**. They were found to be antitubercular, antimicrobial and anti-inflammatory in nature.



(5.23)

This chapter discusses the synthesis and antiamoebic activity of a series of pyrazoline derivatives bearing a tetrazole tail.

5.2. RESULTS AND DISCUSSION

5.2.1. Chemistry

Pyrazoline derivatives bearing a tetrazole tail (Figure 5.24) were synthesized and screened for their probable antiamoebic effects. Target compounds **(1a–15a)** were obtained in a four step reaction procedure as outlined in **Scheme 5.2-5.5**. In the first step, synthesis of chalcones **(1-15)** was carried out by the well-known Claisen-Schmidt reaction and products were purified by recrystallization from methanol (70-85% yield). In the second step, 5-(4-methoxyphenyl)-1*H*-tetrazole **(2)** was synthesized starting from *p*-methoxy benzaldehyde *via* an oxime and nitrile intermediate following the reaction procedure published elsewhere [58, 59] (Scheme 5.3). In the third step 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl]acetohydrazide **(3)** was synthesized starting from 5-(4-methoxyphenyl)-1*H*-tetrazole (**2**) following a reported reaction procedure [60] (Scheme 5.4). The target compounds (**1a-15a**) were obtained by the reaction of chalcones (1-15) and 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl]acetohydrazide (**3**) was refluxed in methanol contaning 5% NaOH **(Scheme 5.5)**. Factors such as the structure and presence of the substituents have profoundly influenced the rate of the reaction. The reaction of haloginated and nitro group substituted chalcones was completed in 10-12 h followed by unsubstituted (15 h), methyl substituted (16-20 h) and alkoxy substituted (24 h) chalcones. The generally accepted interpretation of this reaction, involves the initial formation of an aryl hydrazone with subsequent nucleophilic attack of nitrogen upon the carbon-carbon double bond at β position. Hence the electropositive nature of β carbon may control the overall rate of the reaction. The electropositive nature of β carbon is controlled by the aromatic ring directly attached to it. Halogens and nitro group being electron withdrawing in nature significantly increase the positive character of β carbon lead to

faster reaction while electron donating alkyl and alkoxy groups contributed for slower reaction.

A variety of methods have been reported for the synthesis of 1,3,5-trisubstituted pyrazolines [61-65]. Among the simplest methods, condensation of an α, β-unsaturated ketone with an acid hydrazide in glacial acetic acid (bp 117^{o}C) under reflux was reported by Fischer and Knovenagel [61]. Use of n-butanol (bp 115^{o}C) as reaction medium has significantly improved the yield as reported in some papers. In the present investigation, using n-butanol as a solvent decreased the progress of the reaction and in some cases the reaction did not proceed. The reaction was run in refluxing methanol and 5% NaOH. Structures of compounds **1-15** and **1a-15a** were confirmed by NMR, IR and MS techniques. All of the Pyrazoline derivatives possess similar basic skeletal structure. Proton NMR signals were assigned by comparing the spectra of the products (**1a-15a**) with their corresponding chalcones (**1-15**). Signals around δ value 3.9 and 3.6 ppm recorded as doublet of doublets (dd) were assigned to **4-Ha** and **4-Hb** protons. The *J* values were calculated for above signals and found to be around 18 Hz and 5 Hz for signal around 3.9 ppm and 18 Hz and 11 Hz for signal around 3.6 ppm respectively. 5-H proton (δ around 5.5 ppm) of pyrazoline ring showed a 'dd' pattern of *J* values around 11 Hz and 5 Hz respectively and most likely interacting with 4-Ha and 4-Hb.

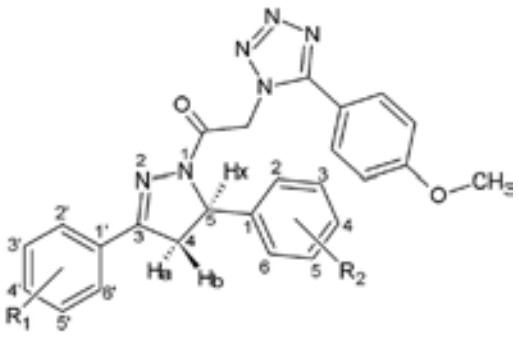
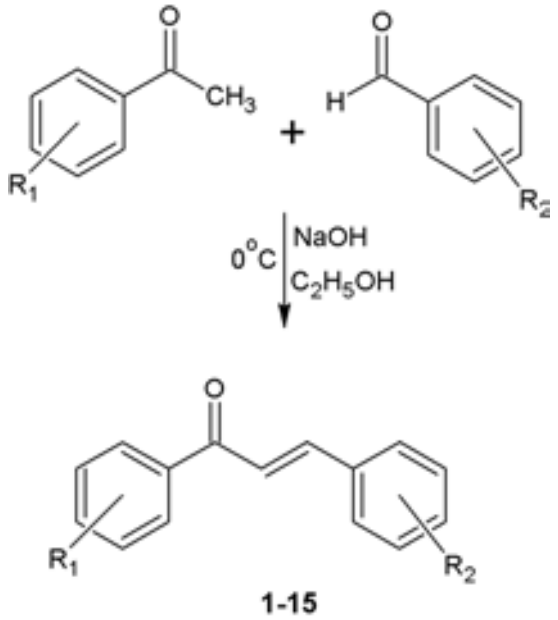
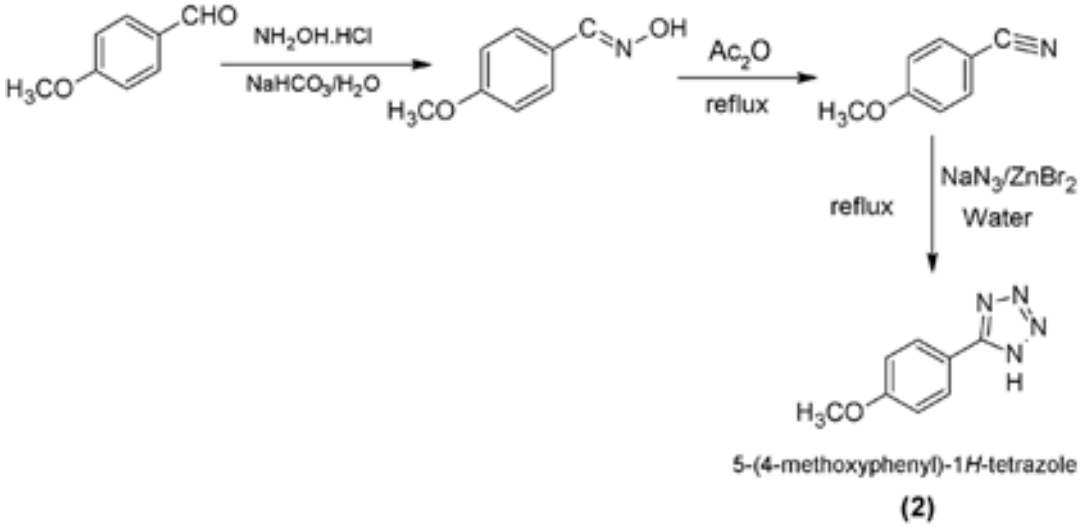


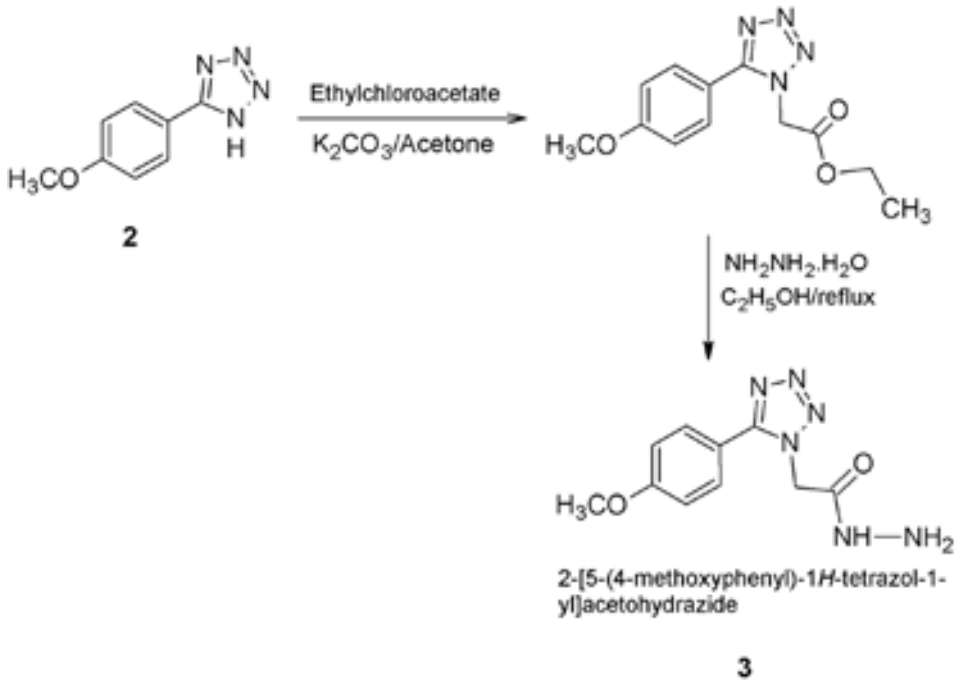
Figure 5.24: Pyrazoline derivatives with a Tetrazole tail.



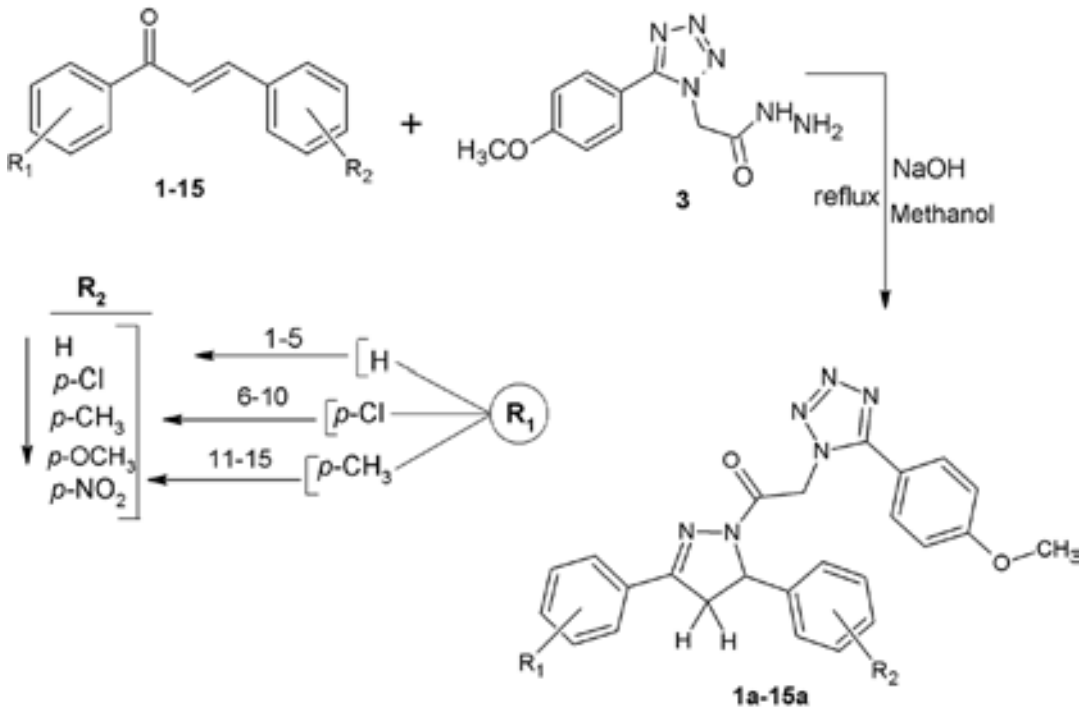
Scheme 5.2: Synthesis of different substituted chalcones.



Scheme 5.3: Synthesis of 5-(4-methoxyphenyl)-1*H*-tetrazole (2)



Scheme 5.4: Synthesis of 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl]acetohydrazide **3**



Scheme 5.5: Synthesis of Pyrazoline derivatives bearing a Tetrazole tail **(1a-15a)**

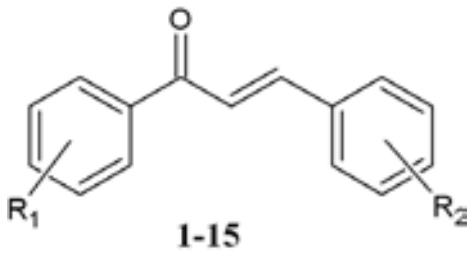
5.2.2. *In vitro* antiamoebic activity

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds (**1-15**) and (**1a-15a)** by microdilution method using HM1: IMSS strain of *Entamoeba histolytica*. The results are summarized in Table 5.1 and 5.2. The data is presented in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with the most widely used antiamoebic medication metronidazole with 50% inhibitory concentration (IC_{50}) of 1.80 μM in our experiments. IC_{50} and 95% confidence limits were interpolated in the corresponding dose response curve. The antiamoebic activity of the test compounds seems to be position and substituent dependent. As depicted in table 5.1 and 5.2 compounds exhibit an interesting inhibition pattern on the tested *HM1:IMSS* strain of *Entamoeba histolytica*. The chalcone derivatives **(1-15)** showed IC_{50} value in the range of 4.19-12.86 μM, which got decreased to 0.86-5.32 μM when the chalcone derivatives were, converted into their corresponding pyrazoline derivatives **(1a-15a)**. The interesting inhibitory behavior of these compounds seems to be dependent on the presence of substituents on the phenyl rings. Compound **1** bearing unsubstituted phenyl ring, showed 50% inhibition at a concentration of 12.86 μM. Presence of substituents overall increased the activity of the tested compounds where compounds bearing electron releasing groups, showed better activity than electron withdrawing group substituted compounds. The pyrazoline derivatives (1a-15a) also showed a similar inhibitory pattern where activity was guided by the presence of electron releasing and electron withdrawing substituents. The results of antiamoebic activity show that the compounds bearing electon withdrawing groups at *para* positions of the phenyl rings show a decreased activity than the compounds

bearing electron donating/releasing groups. It is however important to mention that the activity also depends on the strength of the group present at the position-4 of the phenyl ring. Presence of weakly activating groups like methyl showed less activity than the methoxy group, which is strongly activating. A simplified approach to understand this can be attributed to the electronic effects that substituents can exert. Electron donating groups with lone pairs (OMe) on the atoms adjacent to the p-system activate the aromatic ring by increasing the electron density on the ring through a resonance donating effect. Methyl group without a lone pair however exerts an inductive effect through the s-system due to electronegative effects. Among all the compounds screened compounds bearing a methyl or methoxy group showed better activity than compounds bearing chloro or nitro group at para position of the phenyl rings. Presence of electron releasing and electron withdrawing group in a same compound also resulted in decreased activity. The compound **13a** bearing two methyl groups at para position (6 and 6′) of the phenyl rings showed excellent antiamoebic activity with IC_{50} value of 0.86 µM. The activity however got decreased in compound **14a** in which one of the methyl groups was replaced with a methoxy group. The stronger electron donating group (OCH_3), decreased the activity which corresponds to earlier reports that electron donating groups increase the electron density which makes the compounds effective against microorganisms and enhances their activity [66]. However high electron density causes more difficult diffusion through the cell membrane and substantial activity loss may occur [67]. Thus for a compound an optimum electron density is inevitable so as to gain a significant activity. The compounds 2a, 5a, 7a, 8a, 9a, 10a, 12a and 15a having electron withdrawing chloro and nitro group at *para* position, showed a moderate activity however it was higher than the compound 1a containing an unsubstituted phenyl ring (IC_{50} =5.32 µM).

Based upon the results it will be necessary to optimize the led compounds by substitution at *ortho* and *meta* positions of the phenyl rings by electron releasing/donating or electron withdrawing groups, so that more insight study into the structure activity relationship of such compounds gets revealed. From the results it can be inferred that the compounds **3a**, **4a**, **11a**, **13a** and **14a** showed encouraging results with IC_{50} value in the range of 0.86-1.28 µM, out of which compound **13a** showed the most promising results with 50% inhibitory concentration of 0.86 µM, which is more than two fold better than the reference drug metronidazole (IC_{50} = 1.80 µM). The results were also statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significativity of the difference between the IC_{50} values of metronidazole and the compound 13a was evaluated by *t*-test. The values of the calculated T were found higher than the Table value of T at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

Table 5.1: *In vitro* antiamoebic activity of compounds (1-15) against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile. Compounds with bold font (IC_{50} value) are more active than metronidazole.

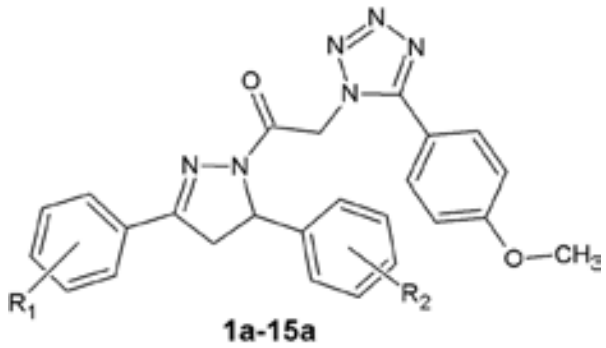


1-15

Comp.	R_1	R_2	Antiamoebic activity		Toxicity Profile	
			IC_{50} (µM)	S.D[a]. (±)	IC_{50} (µM)	Safety Index (SI)
1	H	H	12.86	0.18	>100	>7.78
2	H	Cl	7.32	0.16	97.2	13.27
3	H	CH_3	4.38	0.12	>100	>22.83
4	H	OCH_3	5.93	0.20	>100	>16.86
5	H	NO_2	6.02	0.13	64.7	10.74
6	Cl	H	6.96	0.10	94.7	13.60
7	Cl	Cl	5.86	0.16	82.8	14.13
8	Cl	CH_3	5.32	0.20	92.6	17.40
9	Cl	OCH_3	5.10	0.18	97.2	19.05
10	Cl	NO_2	6.18	0.13	40.2	6.50
11	CH_3	H	4.83	0.14	>100	>20.70
12	CH_3	Cl	5.22	0.16	74.2	14.21
13	CH_3	CH_3	4.19	0.12	>100	23.87
14	CH_3	OCH_3	4.76	0.14	>100	21.01
15	CH_3	NO_2	5.08	0.10	85.6	16.85
MNZ			1.80	0.12	>100	>55.55

[a]The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation. MNZ stands for Metronidazole.

Table 5.2: *In vitro* antiamoebic activity of compounds (1a-15a) against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile. Compounds with bold font (IC_{50} value) are more active than metronidazole.



1a-15a

Comp.	R_1	R_2	Antiamoebic activity		Toxicity Profile	
			IC_{50} (µM)	S.D[a]. (±)	IC_{50} (µM)	Safety Index (SI)
1a	H	H	5.32	0.09	85.3	16.03
2a	H	Cl	3.02	0.12	80.4	26.62
3a	H	CH_3	**1.28**	0.10	**>100**	>78.12
4a	H	OCH_3	**1.16**	0.16	**>100**	>86.20
5a	H	NO_2	3.68	0.10	79.8	21.68
6a	Cl	H	3.15	0.14	90.6	28.76
7a	Cl	Cl	4.65	0.10	80.3	17.26
8a	Cl	CH_3	2.96	0.14	92.6	31.28
9a	Cl	OCH_3	2.63	0.18	>100	>38.02
10a	Cl	NO_2	4.89	0.14	86.5	17.68
11a	CH_3	H	**1.20**	0.18	**>100**	>83.33
12a	CH_3	Cl	2.84	0.16	98.0	34.50
13a	CH_3	CH_3	**0.86**	0.16	**>100**	**>116.27**
14a	CH_3	OCH_3	**1.08**	0.10	>100	>92.59
15a	CH_3	NO_2	2.63	0.13	>100	>38.02
MNZ			1.80	0.12	>100	>55.55

[a]The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation. MNZ stands for Metronidazole.

5.2.3. *In vitro* cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [68, 69] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [70-72].

In the present study, some newly synthesized compounds were screened for their antiamoebic activity and then evaluated for their cytotoxicity against *Human hepatocellular carcinoma cell line (*HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A sub-confluent population of *HepG2* cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13-100 µM. The cell viability (%) obtained with continuous exposure for 48 h are depicted in Figure 5.25. The cytotoxicity of all the compounds was found to be concentration-dependent. **A**ll the compounds including the reference compound metronidazole showed viability in the range of 95-100% at the concentration of 3.13 µM, however the most active antiamoebic compounds showed a vialbility of 100% at this concentration as depicted in Figure 5.25. On increasing the concentration, all the compounds showed a different pattern of cytotoxicity. At a concentration of 25 µM all the compounds showed viability in the range of 72-90%. On increasing the concentration range up to 50 and 100 µM the compounds showed moderate to least cytotoxicity (viability 40-75%). It is also interesting to mention that the highly antiamoebic compounds (**3a**, **4a**, **11a**, **13a** and **14a)** did not show any remarkable cytotoxicity against the *HepG2* cell line. All these

compounds showed a viability of ≥68% at a concentration of 100 μM as depicted in Figure 5.25. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Table 5.1 and 5.2. From the results of antiamoebic activity and cytotoxicity it can be inferred that all the tested compounds **3a**, **4a**, **11a**, **13a** and **14a** are least cytotoxic and excellent *Entamoeba histolytica* inhibitors as compared to the reference drug metronidazole. These results also showed that the compound **13a** despite of being highly antiamoebic do not show any marked toxicity on human cell line and have safety index values of >116.28 which is better than metronidazole (>55.55).

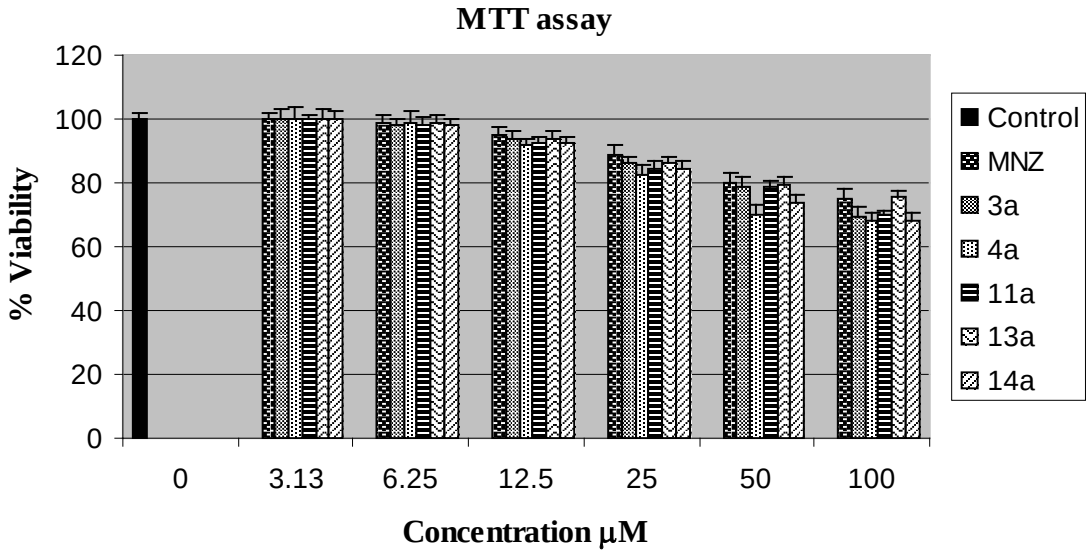


Figure 5.25: Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with Metronidazole, compounds 3a, 4a, 11a, 13a and 14a as evaluated by MTT assay.

5.3. EXPERIMENTAL

5.3.1. Synthesis

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results are uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ± 0.4% of the theoretical values. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-d6/ $CDCl_3$ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapor staining.

5.3.1.1 General method for synthesis of chalcones (1-15)

To the solution of a ketone (Acetophenone, *p*-chloro acetophenone, and *p*-methyl acetophenone 5 mmol) in 5 ml of methanol on an ice bath, freshly prepared 2 N methanolic NaOH solution (30 ml) was added and stirred for 10 min. To this 5 mmole of appropriate aldehyde was added and the reaction mixture was stirred at room temperature for 10-24 h. The reaction mixture was cooled on an ice bath and neutralized with dilute hydrochloric acid. The precipitate appeared was separated by filtration and washed three times with 50 ml distilled water, gave the crude product.

The product so obtained was recrystallized from methanol. The purity of the products was checked on TLC (Merck Silica gel $60F_{254}$) by using mixture of hexane and ethylacetate as mobile phase (7:3v/v).

(2E)-1,3-diphenylprop-2-en-1-one (1): Cream; Yield: 85.3% (3.20 g); mp. 48-50 ^{0}C; Anal. Calc. For $C_{15}H_{12}O$: C 86.51, H 5.81%; found: C 86.38, H 5.69%; IR ν_{max} (cm^{-1}): 3064 (Ar-H), 2939 (C-H), 1658 (C=O), 1460 (C=C); ^{1}HNMR ($CDCl_3$) δ(ppm): 7.89 (m, 10H, Ar-H), 7.70 (d,1H, J= 12 Hz, H-β), 7.58 (d,1H, J= 12 Hz, H-α); ^{13}CNMR($CDCl_3$) δ (ppm): 190.59 (C=O), 144.89 (C-β), (136.90, 132.84, 130.59, 128.67, 127.49, Ar-H), 122.09 (C-α); ESI-MS m/z: $[M+H]^+$ = 209.09.

(2E)-3-(4-chlorophenyl)-1-phenylprop-2-en-1-one (2): White; Yield 87.4% (3.76 g); mp. 83-85 ^{0}C; Anal. Calc. For $C_{15}H_{11}OCl$: C 74.23, H 4.57%; found: C 74.13, H 4.48%; IR ν_{max} (cm^{-1}): 3060 (Ar-H), 2931 (C-H),1662 (C=O),1465 (C=C), 779 (C-Cl); ^{1}HNMR ($CDCl_3$) δ(ppm): 8.05 (d, 2H, *J*= 7.5 Hz, H-2′, H-6′), 7.85 (d, 1H, *J*= 15.6 Hz, H-β), 7.63-7.49 (m, 7H, Ar-H), 7.28 (d, 1H, J= 11.7 Hz, H-α); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.53 (C=O), 143.47 (C-β), (136.45, 135.40, 134.28, 132.41, 130.52, 128.08, Ar-H), 123.54 (C-α); ESI-MS m/z: $[M+H]^+$ =243.05.

(2E)-3-(4-methylphenyl)-1-phenylprop-2-en-1-one (3): White; Yield 95.4% (3.81 g); mp. 67-69 ^{0}C; Anal. Calc. For $C_{16}H_{14}O$: C 86.45, H 6.35 %; found: C 86.33, H 6.24%; IR ν_{max} (cm^{-1}): 3058 (Ar-H), 2932 (C-H), 1659 (C=O), 1447 (C=C); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.05 (d, 2H, *J* = 8.4 Hz, H-2′, H-6′), 7.81 (d, 1H, *J* = 15.6 Hz, H-β), 7.64-7.50 (m, 7H, Ar-H), 7.43 (d, 1H, *J* = 15.6 Hz, H-α), 2.35 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.98 (C=O), 144.54 (C-β), (134.34, 130.12, 129.42, 128.22, phenyl), 123.12 (C-α), 22.48 (methyl); ESI-MS m/z: $[M+H]^+$ = 223.11.

(2E)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one (4): White; Yield 82.8% (3.52 g); mp. 60-63. ^{0}C; Anal. Calc. For $C_{16}H_{14}O_2$: C 80.65, H 5.92%; found: C 80.54, H 5.84%; IR ν_{max} (cm^{-1}): 3054 (Ar-H), 2938 (C-H), 1656 (C=O), 1446 (C=C); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.04 (d, 2H, *J*= 8.1 Hz, H-2′, H-6′), 7.84 (d, 1H, *J*= 15.9 Hz, H-β), 7.64-7.27 (m, 7H, Ar-H), 6.97 (d, 1H, *J*= 15.9 Hz, H-α), 3.88 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ(ppm): 190.64 (C=O), 161.70 (C-β), (144.75, 138.51, 132.60, 130.27, 128.59, 127.62 (Ar-H), 119.78 (C-α), 55.45 (OCH_3); ESI-MS m/z: $M+H^+$= 238.09.

(2E)-3-(4-nitrophenyl)-1-phenylprop-2-en-1-one (5): Orange; Yield 87.7% (3.95 g); mp. 138-140 ^{0}C; Anal. Calc. For $C_{15}H_{11}NO_3$: C 71.14, H 4.38, N 5.53%; found: C 71.26, H 4.35, N 5.69%; IR ν_{max} (cm^{-1}): 3055 (Ar-H), 2923 (C-H), 1668 (C=O), 1460 (C=C), 1547, 1365 (-NO_2); ^{1}H NMR ($CDCl_3$) δ(ppm): 8.31 (d, 2H, *J*= 8.7 Hz, H-2′, H-6′), 8.07 (d, 1H, *J*= 8.4 Hz, H-β), 7.86-7.62 (m, 7H, Ar-H), 7.57 (d, 1H, *J*= 14.7 Hz, H-α); ^{13}C NMR ($CDCl_3$) δ(ppm): 190.10 (C=O), 145.66 (C-β), (136.28, 134.25, 132.06, 128.82, 126.72 (Ar-H), 122.08 (C-α); ESI-MS m/z: $[M+H]^+$ = 254.08.

(2E)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one (6): White; Yield 83% (2.80 g); mp. 81-83 ^{0}C; Anal. Calc. For $C_{15}H_{11}OCl$: C 74.23, H 4.57%; found: C 74.36, H 4.63%; IR ν_{max} (cm^{-1}): 3060 (Ar-H), 2931 (C-H),1662 (C=O),1465 (C=C), 793 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.98 (d, 2H, *J* = 8.4 Hz, H-2′, H-6′), 7.89 (d, 1H, *J* = 15.6 Hz, H- β), 7.66 (d, 1H, J = 15.6 Hz, H-α), 7.51-7.42 (m, 7H, Ar-H); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.89 (C=O), 143.47 (C-β), (136.46, 135.03, 134.31, 130.68, 128.42, Ar-H), 123.18 (C-α); ESI-MS m/z: $[M+H]^+$ = 243.05.

(2E)-1,3-bis(4-chlorophenyl)prop-2-en-1-one (7): White; Yield 90.3% (3.45 g); mp. 96-98 ^{0}C; Anal. Calc. For $C_{15}H_{10}OCl_2$: C 65.01, H 3.64%; found: C 65.18, H 3.50%; IR ν_{max} (cm^{-1}): 3058 (Ar-H), 2930 (C-H),1665 (C=O),1455 (C=C), 778 (C-Cl);

^{1}HNMR ($CDCl_3$) δ (ppm): 7.99 (d, 2H, *J* = 8.4 Hz, H-2′, H-6′), 7.81 (d, 1H, *J* = 15.6 Hz, H- β), 7.61 (d, 1H, *J* = 15.6 Hz, H-α), 7.51-7.49 (m, 2H, H-3′, H-5′), 7.45-7.38 (m, 4H, H-2, H-3, H-5, H-6); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.65 (C=O), 145.47 (C-β), (140.43, 139.24, 136.46, 131.03, 129.68, 128.42, Ar-H), 120.18 (C-α);ESI-MS m/z: $[M+H]^+$= 277.01.

(2E)-1-(4-chlorophenyl)-3-(4-methylphenyl)prop-2-en-1-one (8): White; Yield 86.5% (3.08 g); mp. 158-160 ^{0}C; Anal. Calc. For $C_{16}H_{13}OCl$: C 74.85, H 5.10%; found: C 74.79, H 5.18%; IR ν_{max} (cm^{-1}): 3028 (Ar-H), 2916 (C-H), 1657 (C=O), 1448 (C=C), 793 (C-Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.05 (d, 2H, *J*= 8.4 Hz, H-2′, H-6′), 7.90 (d, 1H, J= 15.6 Hz, H-β), 7.63 (d, 1H, J= 15.6 Hz, H-α), 7.56-7.49 (m, 4H, Ar-H), 7.33-7.27 (m, 2H, Ar-H) 2.47 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.31 (C=O),145.50 (C-β), (141.39, 139.10, 136.64, 131.96, 129.78, 128.93, 128.58, Ar-H), 120.46 (C-α), 21.60 (methyl); ESI-MS m/z: $[M+H]^+$ = 257.07.

(2E)-1-(4-chlorophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (9): White; Yield 98.4% (3.70 g); mp. 118-120 ^{0}C; Anal. Calc. For $C_{16}H_{13}O_2Cl$: C 70.46, H 4.80%; found: C 70.66, H 4.93%; IR ν_{max} (cm^{-1}): 3064 (Ar-H), 2934 (C-H), 1677 (C=O), 1442 (C=C), 790 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.99 (d, 2H, *J*= 12.9 Hz, H-2′, H-6′), 7.84 (d, 1H, *J*= 15.6 Hz, H-β), 7.64-7.47 (m, 4H, Ar-H), 7.41 (d, 1H, *J*= 15.6 Hz, H-β), 6.98 (d, 2H, *J*= 8.7 Hz, H-3, H-5), 3.88 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.22 (C=O), 145.25 (C-β), (138.96, 136.80, 130.37, 129.85, 128.51, 127.42, Ar-H), 119.13 (C-α), 55.46 (OCH_3); ESI-MS m/z: $[M+H]^+$ = 273.06.

(2E)-1-(4-chlorophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (10): Orange; Yield 82.5% (3.27 g); mp. 138-140 ^{0}C; Anal. Calc. For $C_{15}H_{10}NO_3Cl$: C 62.62, H 3.50, N 4.87%; found: C 62.78, H 3.43, N 4.68%; IR ν_{max} (cm^{-1}): 3064 (Ar-H), 2934 (C-H),

1677 (C=O), 1442 (C=C), 1540, 1362 (-NO_2), 789 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.32 (d, 2H, *J*= 12.9 Hz, H-2′, H-6′), 8.02 (d, 1H, *J*= 15.6 Hz, H-β), 7.82 (d, 1H, *J*= 15.6 Hz, H-α), 7.64-7.45 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.37 (C=O), 142.06 (C-β), (135.81, 130.02, 129.20, 129.04, 125.09, Ar-H), 124.29 (C-α); ESI-MS m/z: $[M+H]^+$ = 288.04.

(2E)-1-(4-methylphenyl)-3-phenylprop-2-en-1-one (11): White; Yield 82.2% (2.88 g); mp. 58-60 ^{0}C; Anal. Calc. For $C_{16}H_{14}O$: C 86.45, H 6.35%; found: C 86.63, H 6.48%; IR ν_{max} (cm^{-1}): 3058 (Ar-H), 2932 (C-H), 1659 (C=O), 1447 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.97 (d, 2H, *J*= 7.5 Hz, H-2′, H-6′), 7.85 (d, 1H, *J*= 15.6 Hz, H-β), 7.67-7.53 (m, 4H, Ar-H), 7.44 (d, 1H, *J*= 15.6 Hz, H-α), 7.34-7.27 (m, 3H, Ar-H), 2.46 (s, 3H, CH_3).^{13}C NMR ($CDCl_3$) δ (ppm): 189.29 (C=O), 144.47 (C-β), (134.70, 130.02, 129.53, 128.54, phenyl), 122.58 (C-α), 22.40 (CH_3); ESI-MS m/z: $[M+H]^+$ = 223.11.

(2E)-3-(4-chlorophenyl)-1-(4-methylphenyl)prop-2-en-1-one (12): White; Yield 88.0% (3.60 g); mp. 140-142 ^{0}C; Anal. Calc. For $C_{16}H_{13}OCl$: C 74.85, H 5.10%; found: C 74.70, H 5.18%; IR ν_{max} (cm^{-1}): 3028 (Ar-H), 2916 (C-H), 1657 (C=O), 1448 (C=C), 790 (C-Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.96 (d, 2H, J= 8.1 Hz, H-2′, H-6′), 7.79 (d, 1H, J= 15.6 Hz, H-β), 7.60-7.48 (m, 4H, Ar-H), 7.42 (d, 1H, *J*= 15.6 Hz, H-α), 7.34-7.28 (m, 2H, Ar-H), 2.46 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 184.97 (C=O), 145.44 (C-β), (139.12, 138.15, 131.55, 130.70, 128.75, 124.82, Ar-H), 117.72 (C-α), 16.97 (CH_3); ESI-MS m/z: $[M+H]^+$ = 257.07.

(2E)-1,3-bis(4-methylphenyl)prop-2-en-1-one (13): White; Yield 87.0% (3.30 g); mp. 120-123 ^{0}C; Anal. Calc. For $C_{17}H_{16}O$: C 86.40, H 6.82%; found: C 86.32, H 6.96%; IR ν_{max} (cm^{-1}): 3032 (Ar-H), 2908 (C-H), 1650 (C=O), 1440 (C=C); ^{1}H NMR

($CDCl_3$) δ (ppm): 7.97 (d, 2H, *J*= 8.1 Hz, H-2′, H-6′), 7.84 (d, 1H, *J*= 15.6 Hz, H-β), 7.58-7.49 (m, 4H, Ar-H), 7.33 (d, 1H, *J*= 7.8 Hz, H-α), 7.28-7.23 (m, 2H, Ar-H), 2.46 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.16 (C=O), 144.53 (C-β), (143.54, 140.98, 135.15, 132.25, 129.71, 128.64, 128.47, Ar-H), 121.07 (C-α), 21.71 (CH_3); ESI-MS m/z: $[M^+H]^+$ = 237.12.

(2E)-3-(4-methoxyphenyl)-1-(4-methylphenyl)prop-2-en-1-one (14): White; Yield 85.0% (3.50 g); mp. 98-100 ^0C; Anal. Calc. For $C_{17}H_{16}O_2$: C 80.93, H 6.39%; found: C 80.84, H 6.44%; IR ν_{max} (cm^{-1}): 3025 (Ar-H), 2920 (C-H), 1650 (C=O), 1445 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.96 (d, 2H, *J*= 8.1 Hz, H-2′, H-6′), 7.83 (d, 1H, *J*= 15.6 Hz, H-β), 7.64 (d, 2H, Ar-H), 7.47 (d, 1H, *J*= 15.6 Hz, H-α), 7.33-7.28 (m, 2H, Ar-H), 6.97 (d, 2H, Ar-H), 3.88 (s, 3H, OCH_3), 2.45 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.64 (C=O), 145.04 (C-β), (138.42, 138.05, 132.65, 130.15, 128.55, 124.02, Ar-H), 120.82 (C-α), 54.07 (OCH_3), 21.87 (CH_3); ESI-MS m/z: $[M+H]^+$ = 253.12

(2E)-1-(4-methylphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (15): Orange; Yield 85.0% (3.50 g); mp. 149-151 ^0C, Anal. Calc. For $C_{16}H_{13}NO_3$: C 71.90, H 4.90, N 5.24%; found: C 72.10, H 4.82, N 5.13%; IR ν_{max} (cm^{-1}): 3015 (Ar-H), 2906 (C-H), 1651 (C=O), 1445 (C=C), 1548, 1360 ($-NO_2$); ^1H NMR ($CDCl_3$) δ (ppm): 8.30 (d, 2H, *J*= 8.7 Hz, H-2′, H-6′), 7.98 (d, 1H, *J*= 8.1 Hz, H-β), 7.85 (d, 2H, Ar-H), 7.69 (d, 1H, *J*= 15.6 Hz, H-α), 7.36-7.28 (m, 2H, Ar-H), 6.97 (d, 2H, Ar-H), 2.43 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.12 (C=O), 148.48 (C-β), (144.43, 141.19, 134.99, 129.55, 128.92, 125.76 Ar-H), 124.22 (C-α), 21.77 (CH_3); ESI-MS m/z: $[M+H]^+$ = 268.09.

***5.3.1.2. Synthesis of 5-(4-methoxyphenyl)-1H-tetrazole* (2):** 5-(4-methoxyphenyl)-*1H*-tetrazole was synthesized from 4-methoxybenzonitrile by a reported procedure

[59]. 4-methoxybenzonitrile was obtained from p-methoxy benzaldehyde, by a reported method [58]. Nitrile (2.66 g, 20 mmol), sodium azide (1.43 g, 22 mmol) and zinc bromide (4.50 g, 20 mmol), were refluxed in 60 ml of water. 5 ml of isopropanol was added to stop the formation of clumps. The reaction mixture was refluxed for 24 h and monitored by TLC; vigorous stirring is essential. After 24 h HCl (3 N, 30 ml) and ethyl acetate (100 ml) were added, and vigorous stirring was continued until no solid was present and the aqueous layer had a pH of 1. If necessary, additional ethyl acetate was added. The organic layer was isolated and the aqueous layer extracted with 2 × 100 ml of ethyl acetate. The combined organic layers were evaporated, 200 ml of 0.25 N NaOH was added, and the mixture was stirred for 30 min, until the original precipitate was dissolved and a suspension of zinc hydroxide was formed. The suspension was filtered, and the solid washed with 20 ml of 1 N NaOH. To the filtrate was added 40 ml of 3 N HCl with vigorous stirring causing the tetrazole to precipitate. The tetrazole was filtered and washed with 2 × 20 ml of 3 N HCl and dried in a drying oven to furnish the tetrazole as a white powder.

5.3.1.3. Synthesis of 2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide **(3)**

2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide was prepared by a reported method [60].

5.3.1.4. General method for synthesis of pyrazolines **(1a-15a)**

To the solution of (5 mmol) of the appropriate chalcone (1-15) in 10 ml of methanol and 5% NaOH, (5 mmol, 1.24 g) of *2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide* was added and the reaction mixture was refluxed for 10-24 h. Conversion was monitored after every 60 min interval on precoated silica TLC plates (Merck, $60F_{254}$) by using mixture of Hexane and EtOAc (70:30 v/v) as mobile phase. The excess of solvent was removed under reduced pressure and the reaction mixture

was cooled on an ice bath. The products precipitated out at low temperature were washed five times with 50 ml distilled water, reconstituted in minimum amount of methanol and dried under reduced pressure. In some cases the products were purified by column chromatography using hexane: ethyl acetate (70:30 v/v) as eluent.

1-(4,5-dihydro-3,5-diphenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl) ethanone (1a): White; Yield 45.0%; mp. 290-292 ^{0}C; Anal. Calc. For $C_{25}H_{22}N_6O_2$: C 68.48, H 5.06, N 19.17%; Found: C 68.34, H 5.14, N 19.01%; IR ν_{max} (cm^{-1}): 3035 (Ar-H), 2928 (CH_2), 1683 (C=O), 1592 (C=N), 1446, (C=C), 1238 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.02-6.84 (m, 14H, Ar-H), 5.93 (s, 2H, CH_2), 5.59 [dd,1H, *J* = 11.5, 4.8 Hz, Hx (pyrazoline ring)], 3.87 [dd, 1H, *J* = 18.2, 5.0 Hz, Ha (pyrazoline ring)], 3.32 [dd, 1H, *J*= 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.20 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 154.12 (C=N), 152.85 (C-3, pyrazoline ring), (149.83, 141.35, 132.65, 128.63, 127.68, 127.76, 123.33, 120.34, 114.18, phenyl ring), 55.45 (C-5, pyrazoline ring), 54.56 (OCH_3), 48.51 (CH_2), 42.54 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$= 439.16.

1-(5-(4-chlorophenyl)-4,5-dihydro-3-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl) -1*H*-tetrazol-1-yl)ethanone (2a): White; Yield 45.8%; mp. 238-240 ^{0}C; Anal. Calc. For $C_{25}H_{21}ClN_6O_2$: C 63.49, H 4.48, N 17.77%; found: C 63.58, H 4.34, N 17.81%; IR ν_{max} (cm^{-1}): 3028 (Ar-H), 2930 (CH_2), 1685 (C=O), 1585 (C=N), 1440, (C=C), 1235 (C-N), 790 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.88-6.96 (m, 13H, Ar-H), 5.95 (s, 2H, CH_2), 5.50 [dd,1H, *J* = 11.8, 5.8 Hz, Hx (pyrazoline ring)], 3.80 [dd, 1H, *J* = 18.1, 5.1 Hz, Ha (pyrazoline ring)], 3.38 [dd, 1H, *J*= 18.0, 11.8 Hz, Hb (pyrazoline ring)], 3.28 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.75 (C=O), 155.58 (C=N), 152.80 (C-3, pyrazoline ring), (149.79, 142.90, 133.75, 128.45, 127.25, 127.65,

123.30, 120.46, 114.65, phenyl ring), 56.45 (C-5, pyrazoline ring), 52.10 (OCH_3), 49.55 (CH_2), 42.85 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$ = 473.14.

1-(4,5-dihydro-3-phenyl-5-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl) ethanone (3a): White; Yield 52.5%; mp. 300-302 ^{0}C; Anal. Calc. For $C_{26}H_{24}N_6O_2$: C 69.01, H 5.35, N 18.57%; found: C 69.21, H 5.44 N 18.43%; IR ν_{max} (cm^{-1}): 3035 (Ar-H), 2930 (CH_2), 1682 (C=O), 1585 (C=N), 1448, (C=C), 1235 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.20-7.11 (m, 13H, Ar-H), 5.87 (s, 2H, CH_2), 5.58 [dd,1H, *J* = 11.0, 5.4 Hz, Hx (pyrazoline ring)], 3.85 [dd, 1H, *J* = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.32 [dd, 1H, *J*= 11.8, 14.6 Hz, Hb (pyrazoline ring)], 3.24 (s, 3H, OCH_3), 2.23 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 154.88 (C=N), 150.44 (C-3, pyrazoline ring), (148.09, 142.34, 133.05, 128.24, 127.15, 126.45, 123.38, 120.25, 114.25, phenyl ring), 56.35 (C-5, pyrazoline ring), 54.80 (OCH_3), 48.55 (CH_2), 44.65 (C-4, pyrazoline ring), 22.85 (CH_3); ESI-MS m/z: $[M+H]^+$ = 453.20.

1-(4,5-dihydro-5-(4-methoxyphenyl)-3-phenylpyrazol-1-yl)-2-(5-(4-methoxy phenyl)-1*H*-tetrazol-1-yl)ethanone (4a): White; Yield 48.0%; mp. 198-200 ^{0}C; Anal. Calc. For $C_{26}H_{24}N_6O_3$: C 66.65, H 5.16, N 17.94%; found: C 66.53, H 5.08, N 17.83%; IR ν_{max} (cm^{-1}): 3025 (Ar-H), 2924 (CH_2), 1685 (C=O), 1580 (C=N), 1442, (C=C), 1230 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.98-6.88 (m, 14H, Ar-H), 5.87 (s, 2H, CH_2), 5.53 [dd,1H, *J* = 11.6, 5.0 Hz, Hx (pyrazoline ring)], 3.67 [dd, 1H, *J* = 18.8, 5.4 Hz, Ha (pyrazoline ring)], 3.40 [dd, 1H, *J*= 18.0, 11.5 Hz, Hb (pyrazoline ring)], 3.33 (s, 3H, OCH_3), 3.25 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.46 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 54.38 (C-5, pyrazoline ring),

52.16 (OCH_3), 49.50 (CH_2), 44.45 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$ = 469.19.

1-(4,5-dihydro-5-(4-nitrophenyl)-3-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (5a): Yellowish; Yield 54.8%; mp. 298-300 ^{0}C; Anal. Calc. For $C_{25}H_{21}N_7O_4$: C 62.11, H 4.38, N 20.28%; found: C 62.32, H 4.26, N 20.37%; IR ν_{max} (cm^{-1}): 3038 (Ar-H), 2921 (CH_2), 1682 (C=O), 1580 (C=N), 1440, (C=C), 1230 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.10-7.04 (m, 13H, Ar-H), 5.93 (s, 2H, CH_2), 5.52 [dd,1H, *J* = 11.4, 4.9 Hz, Hx (pyrazoline ring)], 3.87 [dd, 1H, *J* = 18.1, 4.8 Hz, Ha (pyrazoline ring)], 3.30 [dd, 1H, *J*= 18.5, 11.7 Hz, Hb (pyrazoline ring)], 3.24 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.48 (C=O), 154.87 (C=N), 152.88 (C-3, pyrazoline ring), (149.10, 142.76, 133.28, 129.80, 127.42, 126.21, 123.20, 120.10, 114.35, phenyl ring), 56.45 (C-5, pyrazoline ring), 54.65 (OCH_3), 48.45 (CH_2), 43.28 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$ = 484.17.

1-(3-(4-chlorophenyl)-4,5-dihydro-5-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (6a): White; Yield 58.0%; mp. 310-312 ^{0}C; Anal. Calc. For $C_{25}H_{21}ClN_6O_2$: C 63.49, H 4.48, N 17.77%; found: C 63.35, H 4.54, N 17.83%; IR ν_{max} (cm^{-1}): 3033 (Ar-H), 2928 (CH_2), 1682 (C=O), 1585 (C=N), 1437, (C=C), 1236 (C-N), 793 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.08-6.91 (m, 13H, Ar-H), 5.87 (s, 2H, CH_2), 5.55 [dd,1H, *J* = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.83 [dd, 1H, J = 18.2, 5.1 Hz, Ha (pyrazoline ring)], 3.40 [dd, 1H, *J*= 11.8, 14.4 Hz, Hb (pyrazoline ring)], 3.26 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 55.38 (C-5, pyrazoline ring), 54.10 (OCH_3), 48.50 (CH_2), 46.45 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$ = 473.14.

1-(3,5-bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (7a): Cream; Yield 61.5%; mp. 199-201 ^{0}C; Anal. Calc. For $C_{25}H_{20}Cl_2N_6O_2$: C 59.18, H 3.97, N 16.56%; found: C 59.26, H 3.87, N 16.43%; IR ν_{max} (cm^{-1}): 3025 (Ar-H), 2932 (CH_2), 1680 (C=O), 1582 (C=N), 1435, (C=C), 1230 (C-N), 790 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.07-6.98 (m, 12H, Ar-H), 5.93 (s, 2H, CH_2), 5.53 [dd,1H, *J* = 11.5, 4.5 Hz, Hx (pyrazoline ring)], 3.87 [dd, 1H, *J* = 18.0, 4.8 Hz, Ha (pyrazoline ring)], 3.37 [dd, 1H, *J*= 11.4, 14.6 Hz, Hb (pyrazoline ring)], 3.28 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.35 (C=O), 154.28 (C=N), 152.10 (C-3, pyrazoline ring), (148.25, 142.05, 131.20, 128.64, 127.60, 126.11, 123.10, 120.82, 114.45, phenyl ring), 56.45 (C-5, pyrazoline ring), 54.02 (OCH_3), 49.65 (CH_2), 43.25 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$=507.10.

1-(3-(4-chlorophenyl)-4,5-dihydro-5-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (8a): White; Yield 55.0%; mp. 208-210 ^{0}C; Anal. Calc. For $C_{26}H_{23}ClN_6O_2$: C 64.13, H 4.76, N 17.26%; found: C 64.25, H 4.65, N 17.10%; IR ν_{max} (cm^{-1}): 3032 (Ar-H), 2935 (CH_2), 1682 (C=O), 1588 (C=N), 1445, (C=C), 1230 (C-N), 779 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.07-6.98 (m, 12H, Ar-H), 5.87 (s, 2H, CH_2), 5.55 [dd,1H, *J* = 11.6, 4.9 Hz, Hx (pyrazoline ring)], 3.83 [dd, 1H, *J* = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.40 [dd, 1H, *J*= 18.0, 11.6 Hz, Hb (pyrazoline ring)], 3.26 (s, 3H, OCH_3), 2.24 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 58.38 (C-5, pyrazoline ring), 52.10 (OCH_3), 48.50 (CH_2), 44.85 (C-4, pyrazoline ring), 22.65 (CH_3); ESI-MS m/z: $[M+H]^+$= 487.16.

1-(3-(4-chlorophenyl)-4,5-dihydro-5-(4-methoxyphenyl)pyrazol-1-yl)-2-(5-(4-methoxy phenyl)-1*H*-tetrazol-1-yl)ethanone (9a): Yellowish; Yield 65.0%; mp.

315-317 ^{0}C; Anal. Calc. For $C_{26}H_{23}ClN_6O_3$: C 62.09, H 4.61, N 16.71%; found: C 62.31, H 4.47, N 16.78%; IR ν_{max} (cm^{-1}): 3025 (Ar-H), 2932 (CH_2), 1680 (C=O), 1582 (C=N), 1445, (C=C), 1233 (C-N), 778 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.87-6.48 (m, 12H, Ar-H), 5.89 (s, 2H, CH_2), 5.58 [dd, 1H, *J* = 11.5, 4.7 Hz, Hx (pyrazoline ring)], 3.88 [dd, 1H, *J* = 18.4, 5.4 Hz, Ha (pyrazoline ring)], 3.64 [dd, 1H, *J*= 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.34 (s, 3H, OCH_3), 3.26 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.85 (C=O), 156.45 (C=N), 152.65 (C-3, pyrazoline ring), (148.25, 143.65, 133.20, 128.54, 127.33, 125.89, 123.45, 120.25, 114.28, phenyl ring), 56.40 (C-5, pyrazoline ring), 50.65 (OCH_3), 46.58 (CH_2), 45.35 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$= 503.15.

1-(3-(4-chlorophenyl)-4,5-dihydro-5-(4-nitrophenyl)pyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (10a): Orange red; Yield 52.3%; mp. 187-190 ^{0}C; Anal. Calc. For $C_{25}H_{20}ClN_7O_4$: C 57.98, H 3.89, N 18.93%; found: C 57.83, H 3.94, N 18.80%; IR ν_{max} (cm^{-1}): 3035 (Ar-H), 2934 (CH_2), 1685 (C=O), 1580 (C=N), 1445, (C=C), 1230 (C-N), 792 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.98-6.78 (m, 13H, Ar-H), 5.84 (s, 2H, CH_2), 5.48 [dd,1H, *J* = 11.5, 5.0 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, *J* = 17.8, 5.0 Hz, Ha (pyrazoline ring)], 3.67 [dd, 1H, *J*= 17.8, 11.7 Hz, Hb (pyrazoline ring)], 3.30 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.20 (C=O), 154.98 (C=N), 151.35 (C-3, pyrazoline ring), (149.55, 144.15, 131.28, 128.34, 127.03, 125.09, 123.65, 120.18, 114.28, phenyl ring), 56.48 (C-5, pyrazoline ring), 55.06 (OCH_3), 48.24 (CH_2), 43.08 (C-4, pyrazoline ring); ESI-MS m/z: $[M+H]^+$= 518.13.

1-(4,5-dihydro-5-phenyl-3-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl) ethanone (11a): White; Yield 60.0%; mp. 302-305 ^{0}C; Anal. Calc. For $C_{26}H_{24}N_6O_2$: C 69.01, H 5.35, N 18.57%; found: C 69.13, H 5.19, N 18.48%; IR ν_{max}

(cm^{-1}): 3035 (Ar-H), 2932 (CH_2), 1682 (C=O), 1580 (C=N), 1435, (C=C), 1230 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.01-6.93 (m, 13H, Ar-H), 5.90 (s, 2H, CH_2), 5.54 [dd,1H, *J* = 11.2, 4.8 Hz, Hx (pyrazoline ring)], 3.89 [dd, 1H, *J* = 17.4, 4.7 Hz, Ha (pyrazoline ring)], 3.58 [dd, 1H, *J* = 17.8, 11.2 Hz, Hb (pyrazoline ring)], 3.32 (s, 3H, OCH_3), 2.28 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.20 (C=O), 154.98 (C=N), 151.35 (C-3, pyrazoline ring), (149.55, 144.15, 131.28, 128.34, 127.03, 125.09, 123.65, 120.18, 114.28, phenyl ring), 58.48 (C-5, pyrazoline ring), 55.45 (OCH_3), 48.24 (CH_2), 43.08 (C-4, pyrazoline ring), 20.45 (CH_3); ESI-MS m/z: $[M+H]^+$ = 453.20.

1-(5-(4-chlorophenyl)-4,5-dihydro-3-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H* tetrazol-1-yl)ethanone (12a): Yellowish; Yield 47.0%; mp. 210-213 ^{0}C; Anal. Calc. For $C_{26}H_{23}ClN_6O_2$: C 64.13, H 4.76, N 17.26%; found: C 64.27, H 4.62, N 17.18%; IR ν_{max} (cm^{-1}): 3032 (Ar-H), 2935 (CH_2), 1680 (C=O), 1580 (C=N), 1445, (C=C), 1230 (C-N), 790 (C-Cl); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.09-6.91 (m, 12H, Ar-H), 5.88 (s, 2H, CH_2), 5.56 [dd,1H, *J* = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, *J* = 17.8, 4.8 Hz, Ha (pyrazoline ring)], 3.56 [dd, 1H, *J* = 18.0, 11.8 Hz, Hb (pyrazoline ring)], 3.38 (s, 3H, OCH_3), 2.32 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.28 (C=O), 154.06 (C=N), 152.30 (C-3, pyrazoline ring), (149.25, 144.29, 131.25, 128.15, 127.16, 125.04, 123.45, 120.45, 114.45, phenyl ring), 58.25 (C-5, pyrazoline ring), 55.05 (OCH_3), 48.20 (CH_2), 42.64 (C-4, pyrazoline ring) 22.60 (CH_3); ESI-MS m/z: $[M+H]^+$= 487.16.

1-(4,5-dihydro-3,5-di*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl)ethanone (13a): White; Yield 56.2%; mp. 149-151 ^{0}C, Anal. Calc. For $C_{27}H_{26}N_6O_2$: C 69.51, H 5.62, N 18.01%; found: C 69.34, H 5.52, N 18.13%; IR ν_{max} (cm^{-1}): 3025 (Ar-H), 2925 (CH_2), 1682 (C=O), 1585 (C=N), 1445, (C=C), 1238 (C-

N); ^{1}HNMR ($CDCl_3$) δ (ppm): 7.98-6.65 (m, 12H, Ar-H), 5.90 (s, 2H, CH_2), 5.58 [dd,1H, *J* = 11.5, 5.0 Hz, Hx (pyrazoline ring)], 3.84 [dd, 1H, *J* = 18.1, 5.1 Hz, Ha (pyrazoline ring)], 3.54 [dd, 1H, *J* = 17.4, 11.6 Hz, Hb (pyrazoline ring)], 3.34 (s, 3H, OCH_3), 2.27 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.65 (C=O), 153.28 (C=N), 151.30 (C-3, pyrazoline ring), (149.25, 143.18, 131.25, 128.14, 127.63, 124.45, 123.25, 120.48, 114.20, phenyl ring), 56.40 (C-5, pyrazoline ring), 54.85 (OCH_3), 48.25 (CH_2), 42.68 (C-4, pyrazoline ring), 22.40 (CH_3); ESI-MS m/z: $[M+H]^+$= 467.21.

1-(4,5-dihydro-5-(4-methoxyphenyl)-3-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxy-phenyl)-1*H* tetrazol-1-yl)ethanone (14a): Cream; Yield 45.0%; mp. 149-151 ^{0}C, Anal. Calc. For $C_{27}H_{26}N_6O_3$: C 67.21, H 5.43, N 17.42%; found: C 67.28, H 5.40, N 17.34%; IR ν_{max} (cm^{-1}): 3032 (Ar-H), 2925 (CH_2), 1682 (C=O), 1580 (C=N), 1435, (C=C), 1232 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.10-6.98 (m, 12H, Ar-H), 5.88 (s, 2H, CH_2), 5.58 [dd,1H, *J* = 11.6, 5.0 Hz, Hx (pyrazoline ring)], 3.93 [dd, 1H, *J* = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.59 [dd, 1H, *J* = 18.0, 11.5 Hz, Hb (pyrazoline ring)], 3.38 (s, 3H, OCH_3), 3.30 (s, 3H, OCH_3), 2.24 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.27 (C=O), 158.48 (C=N), 152.65 (C-3, pyrazoline ring), (149.44, 142.20, 131.20, 128.11, 127.18, 125.04, 123.25, 120.15, 114.25, phenyl ring), 56.40 (C-5, pyrazoline ring), 54.16 (OCH_3), 46.25 (CH_2), 42.15 (C-4, pyrazoline ring), 21.80 (CH_3); ESI-MS m/z: $[M+H]^+$= 483.21.

1-(4,5-dihydro-5-(4-nitrophenyl)-3-*p*-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1*H*-tetrazol -1-yl)ethanone (15a): Orange red; Yield 46.5%; mp. 149-151 ^{0}C, Anal. Calc. For $C_{26}H_{23}N_7O_4$: C 62.77, H 4.66, N 19.71%; found: C 62.54, H 4.74, N 19.57%; IR ν_{max} (cm^{-1}): 3035 (Ar-H), 2930 (CH_2), 1680 (C=O), 1582 (C=N), 1441, (C=C), 1230 (C-N); ^{1}HNMR ($CDCl_3$) δ (ppm): 8.09-6.93 (m, 12H, Ar-H), 5.90 (s, 2H,

CH_2), 5.54 [dd,1H, *J* = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.87 [dd, 1H, *J* = 17.8, 4.8 Hz, Ha (pyrazoline ring)], 3.55 [dd, 1H, *J* = 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.36 (s, 3H, OCH_3), 2.18 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.85 (C=O), 156.15 (C=N), 152.45 (C-3, pyrazoline ring), (149.28, 142.26, 131.25, 128.45, 127.25, 125.26, 123.25, 120.55, 114.20, phenyl ring), 58.28 (C-5, pyrazoline ring), 55.50 (OCH_3), 46.20 (CH_2), 42.05 (C-4, pyrazoline ring), 24.11 (CH_3); ESI-MS m/z: $[M+H]^+$= 498.18.

5.3.2. *In vitro* antiamoebic assay

All the compounds **(1-15 and 1a-15a)** were screened *in vitro* for antiamoebic activity against *HM1:IMSS* strain of *Entamoeba histolytica* by microdilution method [73]. The detailed procedure is given in Chapter 2.

5.3.3. Cytotoxicity studies (MTT assay)

5.3.3.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37^0C in a saturated humidity atmosphere containing 95% air/5% CO_2 [74]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

5.3.3.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [75]. The detailed procedure is given in Chapter 2.

5.4. Conclusion

This study achieved the synthesis of a series of pyrazoline derivatives (1a-15a) from their parent α, β unsaturated ketones (1-15). This study confirmed the great potential of the class of pyrazoline compounds bearing a tetrazole tail as innovative antiamoebic compounds (IC_{50} = 0.86-5.32 µM) as compared to their parent chalcone derivatives (IC_{50} =4.19-12.86 µM). Through a preliminary SAR campaign, it was found that presence of substituents play a dominant role in the activity of the compounds, where activity seems to be guided by the electron donating and withdrawing nature of the substituents. Compound **13a** bearing methyl group at *para* position of the phenyl rings happened to be the most potent of all the compounds reported here. The replacement of methyl group with a stronger electron releasing methoxy group resulted in decrease in activity. The results revealed that an optimum electron density is inevitable for a compound to gain a significant activity. Compound **3a**, **4a**, **11a**, and **14a** also showed better activity (IC_{50} =1.08-1.28 µM) than the reference compound metronidazole (IC_{50} =1.80 µM). Cytotoxicity studies on human hepatocellular carcinoma cell line *HepG2* also revealed non cytotoxic nature of these active compounds. The most promising results were observed for compound **13a** (*1-(4,5-dihydro-3,5-dip-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone*) with potent antiamoebic activity (IC_{50} = 0.86 µM), least cytotoxicity (IC_{50} = >100 µM) and safety index value of >116.28, which is better than metronidazole (>55.55).

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Chapter 6

Synthesis, characterization, nanosizing and in vitro antiamoebic evaluation of acyl hydrazone ligand based Cu(II), Co(II) and Ni(II) complexes

6.1. INTRODUCTION

"Nanotechnology" can be broadly defined as the creation of objects and surfaces whose unique functions are the direct result of the nanoscale dimensions and/or organization. These unique properties may be mechanical, electrical, or photochemical and are not seen in the bulk materials. The prefix comes from the ancient Greek *νᾶνος* through the Latin *nanus* meaning literally *dwarf* and, by extension, *very small*. Within the convention of International System of Units (SI) it is used to indicate the reduction factor of 10^{-9} times. Nanotechnology manipulates matter at the scale of one billionth of a meter (10^{-9} m). It is more of an approach to engineering than a science, although it draws on the scientific knowledge of biology, physics, chemistry, and materials science and is expected to change these sciences dramatically. **Eric Drexler** introduced the term "nanotechnology" in Engines of Creation (1986) to describe the "manipulation of individual atoms and molecules to build structures to complex, atomic specifications" and stated that "perhaps the arrival of the concept of nanotechnology came about in physicist Richard Feynman's landmark 1959 lecture called There's Plenty of Room at the Bottom"[1].

"Nanoscale" generally refers to objects 1 -100 nm in one or more dimensions. At its lower limit this definition intentionally excludes individual molecules which generally define the lower end of the nanotechnology, i.e. nano derived features are as much a function of larger bulk materials approaching a molecular scale as they are a selective change in molecule's properties as they aggregate [2]. The inorganic nanoparticles have been prepared since the first prepositions made by S. Friberg and F. Gault. These ideas were followed by a rapid increase in original research works related to the preparation of metal and metal boride nanoparticles [3]. Nanometer scale particles composed of metals, metal oxides and other inorganic materials have been reported in

vast majority [4, 5]. However the preparation of nanoparticles from general organic molecules has been paid little attention. Organic nanoparticles of β-carotene, perylene, polydiacetylene, pyrazolines, cholesterol, rhovanil, retinol and rhodiorome have been prepared [6, 7]. Because of the diversity of organic molecules there is tendency to extend the research on nanoparticles from metals and semiconductors into the general organic field.

Only a limited number of organic nanoparticles can be prepared using oil in water microemulsions usually called microemulsion polymerization [8]. Nanosize polymer particles can be obtained using polymerization reactions in o/w microemulsions, this leads to hydrophobic nanoparticles (10-40nm) dispersed in water [9]. The advantage of this method is fast polymerization rates and high molar mass of polymers, while the drawback is the need of high weight ratio of surfactant to polymer. The first successful microemulsion polymerization was reported by Atik and Thomas 1981 who used CTAB/styrene/hexanal/water O/W microemulsion [10]. The reaction was carried out either thermally using azobisisobutyronitrile (AIBN) or radiolytically using Cs γ- ray source. Monodisperse latex nanoparticles of diameters 35 and 20 nm were obtained, respectively. Styrene has also been polymerized using three component microemulsions of dodecyl trimethyl ammonium bromide (DTAB) and potassium persulphate (KPS) initiator [11]. This resulted in monodisperse lattices with radii in the range of 20-30nm. Guo, et al., [12] studied styrene polymerization in SDS/pentanol/ water microemulsions using both water soluble KPS and oil soluble AMBN as initiators and found that the fraction of formed particles were determined by the amount of initiator. Palani, et al., [13] studied the polymerization of MMA using MMA/ethylene glycol dimethacrylate/water system with acylamide as

amphiphile. The particles formed were transparent up to 60% of water in the microemulsion systems.

Different microemulsion systems have been used to synthesize organic nanoparticles of cholesterol, Retinol Rhodiarome, Rhovanil [14]. The microemulsions used are AOT/heptane/water, Triton/decanol/water, and CTABr/ hexanol/water. The general preparation of these organic nanoparticles consists of the direct precipitation of the active compound in the aqueous cores of the microemulsion. After their preparation, nanoparticles are revealed with iodine vapor and observed with a transmission electron microscope [15]. This consists of several stages. The solution of the active compound in an appropriate solvent penetrates inside the aqueous cores by crossing the interfacial film. The solvent certainly plays a role in the transport of the active compound inside the aqueous cores. The active compound precipitates in the aqueous cores because of its insolubility in water, and the nuclei are thus formed. The so formed nuclei can grow because of the exchange of the active compound between the aqueous cores. Finally, the nanoparticles are stabilized by the surfactants.

Solid nanoparticles of nimesulide (molecule of a pharmaceutical interest) have been synthesized by direct precipitation in two water/oil (W/O) microemulsion systems Epikuron 170 (E170, which is a lecithin)/isopropyl myristate/water/*n*-butanol (ME1) and E170/isopropyl myristate/water/isopropanol (ME2). The size distributions of the observed nanoparticles are relatively narrow for the two systems. In the two microemulsions, the diameter of the nanoparticles is between 45 and 60 A°. The interest in these organic nanoparticles lies in their pharmaceutical applications [16]. First, the microemulsions used to synthesize the nanoparticles are potential systems for drug delivery [17]. Indeed, microemulsions have a low viscosity, making intravenous injections easier. Second, the solid organic substances could be injected

directly into the vena in the form of nanoparticles. As these substances are often insoluble in water, a classical method of drug delivery using aqueous solutions is not applicable. However, if nanoparticles could be prepared in suspension in water, they could be directly injected. The size of the particles is very important, because bigger particles could lead to embolism.

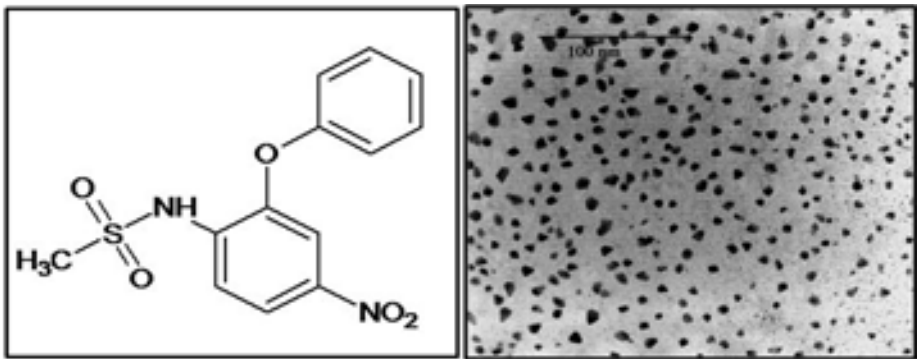


Figure 6.1: Molecular structure and TEM image of Nimesulide nanoparticles.

In another study solid nanoparticles of Amoxicillin have been reported. Three different types of amoxicillin nanostructures were synthesized successfully [18]. It is conceivable that these special nanostructures can enhance the solubility rate of dissolution, and bioactivity of amoxicillin [19].

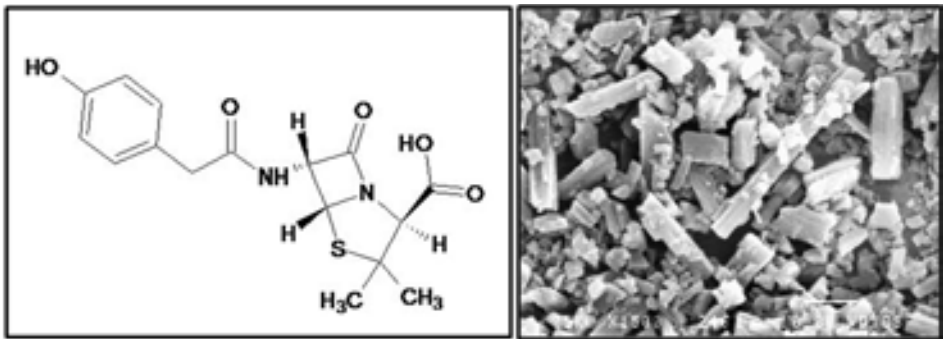
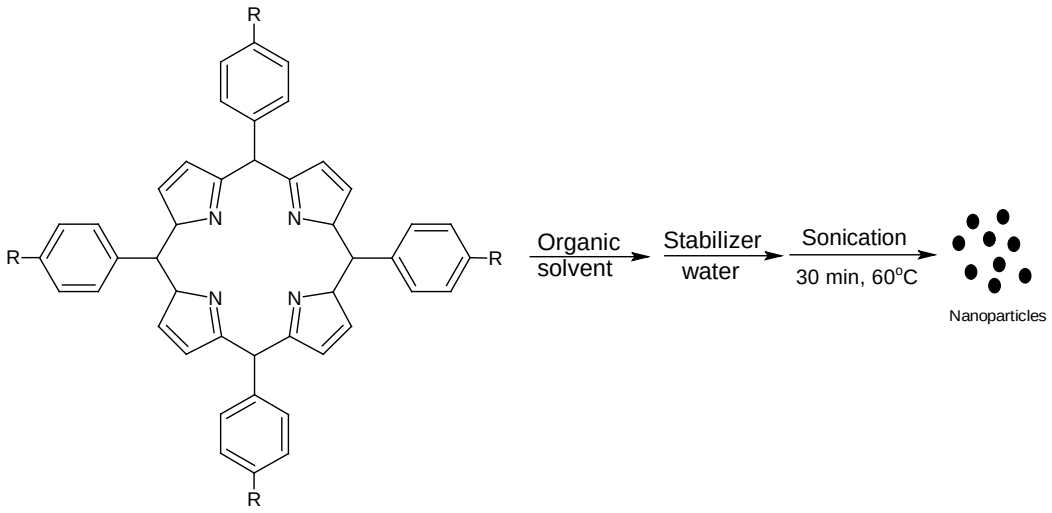


Figure 6.2: Molecular structure and SEM image of amoxicillin nanoparticles.

M.M.K.Motlagh et al., report the synthesis and optical properties of organic porphyrin nanoparticles with narrow size distribution and well dispersibility [20]. Nanoparticles were produced by a combination of the reprecipitation and sonication method and termed the "ultrasonic method". The porphyrin nanoparticles were stable in solution without precipitation for at least 30 days. No self-aggregation of the constituent porphyrin chromophores was confirmed. The porphyrin nanoparticles exhibited interesting optical properties.



Scheme 6.1: Synthesis of Porphyrin nanoparticles

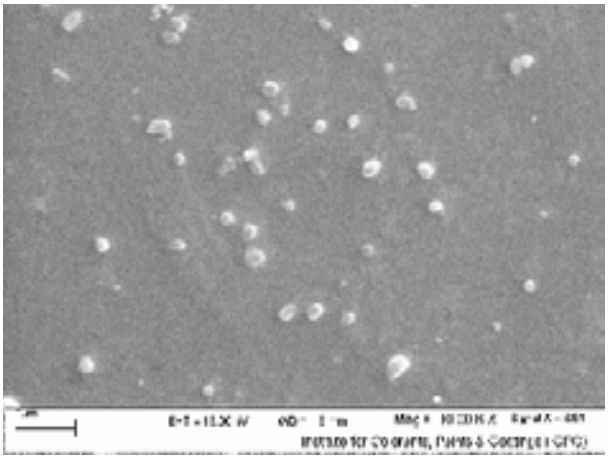


Figure 6.3: SEM image of porphyrin nanoparticles

This chapter discusses the synthesis, nanosizing and antiamoebic activity of acyl-hydrazone ligand based Cu(II), Co(II) and Ni(II) complexes.

6.2. RESULTS AND DISCUSSION

6.2.1. Chemistry

The study of complexes formed with hydrazones is very interesting because of the tautomerism exhibited by these ligands. The chromophores group –CO-NH-N< of these ligands can enter the inner core of the complexes either in keto or enol form. In the keto form they behave as monobasic tridentates and whereas in enol form they behave as dibasic tridentates. This tendency depends on a number of factors such as pH of the medium employed in the synthesis of the complexes, the nature of the substituents attached to the carbonyl carbon atom, β nitrogen atom, anion of the metal salt and the metal.

The resulting double bond $–N^2=C^3H–$ following the condensation reaction, contributes to the formation of geometrical isomers Z and E for the ligand (L). Geometrical isomerism may have some important role in the bioactivity of the acyl-hydrazones hence their studies are very crucial to develop synthetic methods for selective synthesis of a particular isomer. Electrospray mass spectra showed high purity of the acylhydrazone. In the NMR spectra, one distinctive feature is the amide proton resonance which is well downfield of all other peaks. Thus, the ^1H NMR spectra show an intense singlet signal at 12.08 ppm assigned to N^1H proton. This greater deshielding effect is most likely caused by intramolecular H bonding of the type seen in the solid-state structure of the Z isomer (Figure 6.4) [21]. The distinctly different proton chemical shifts of 2-(5-methyl-2-nitro-1H-imidazol-1-yl)-N'-[(E)-pyridin-2-yl methylidene]acetohydrazide (L) indicate that this chelator adopt an E isomeric form in solution [22, 23].

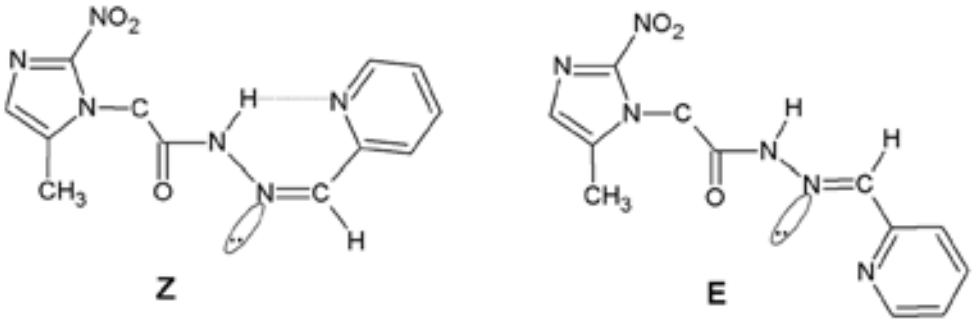


Figure 6.4. *Interconversion of Z and E isomers of the ligand (L).*

MM2 calculation [24] indicated that the minimum energy stabilization of E isomer (-1.25 kcal/mol) and heat of formation (157.94 kcal/mol) were lower than that of Z isomer (-0.76 kcal/mol and 308.16 kcal/mol, respectively). Hence E isomer should form predominantly. Ligand presents keto-enol tautomerism, which is manifested especially in solution by proton migration from hydrazine nitrogen atom N^1H to neighbor carbonyl oxygen atom and formation of imide bond (Figure 6.5). The ability of this ligand to coordinate a metal ion is influenced by changing the configuration and conformation, stability in different working conditions and especially by the density of negative charge, which can be partially transferred during the process of complexation of metal ion.

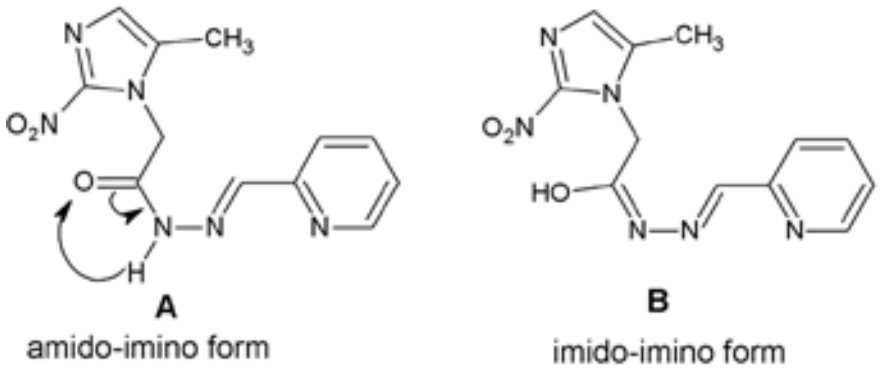


Figure 6.5: Tautomerism of *2-(5-methyl-2-nitro-1H-imidazol-1-yl)-N'-[(E)-pyridin-2-yl methyldene] acetohydrazide* **(L)**

Using molecular orbital method (Huckel calculation) [24] the charge density per heteroatom was determined (Figure 6.6). The obtained values show that the donor site is on the fragment –CO–N^1H–N^2=CH– and the ligand coordinate to the metal ions into E isomeric form. In this form, the pyridyl radical has a good geometric position in order to bind metal ion by non-bonding electron doublet of nitrogen atom. In these conditions, in obtained complex combinations, the metal ion can adopt different geometries, due to the capacity of *2-(5-methyl-2-nitro-1H-imidazol-1-yl)-N'-[(E)-pyridin-2-ylmethylidene] acetohydrazide* (L) to act as a bidentate or tridentate ligand.

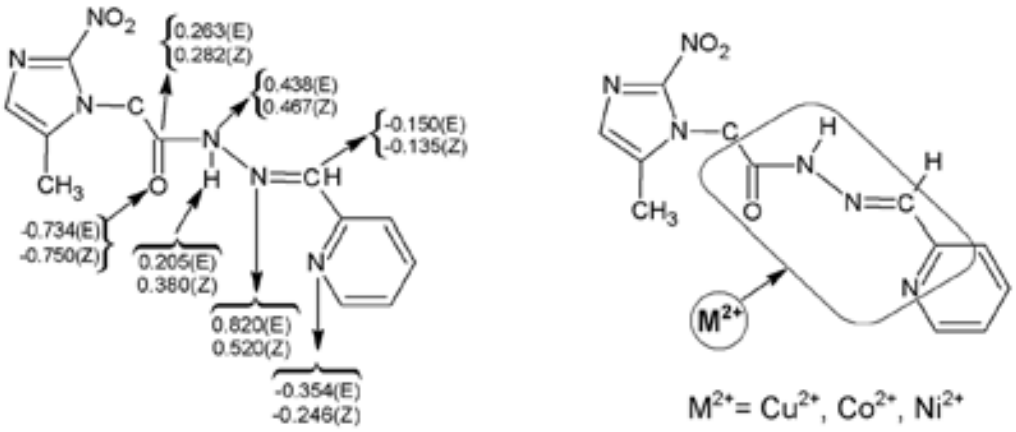


Figure 6.6: The charge density per heteroatom and the donor site of *2-(5-methyl-2-nitro-1H-imidazol-1-yl)-N'-[(E)-pyridin-2-ylmethyldene]acetohydrazide* **(L)**

The obtained complexes are microcrystalline solids which are stable in air, are soluble in DMF and DMSO, but insoluble in other organic solvents and have melting points higher than 300^{o}C. The molar conductance values in DMSO (10^{-3} M) are too low to account for any dissociation, therefore the complexes are considered to be non-electrolytes [25]. The elemental analyses data along with some physical properties of the ligand and its complexes are reported in Table 6.1.

Table 6.1: Analytical data and physical properties of 2-(5-methyl-2-nitro-1H-Imidazol-1-yl)-N'-[(E)-pyridin-2-ylmethylidene]acetohydrazide (L) and its Cu(II), Co(II) and Ni(II) complexes.

Comp.	Molecular formulae	MW ($gmol^{-1}$)	Colour	Elemental analysis calc. (expt.)			μ (BM)
				C	H	N	
L	$C_{12}H_{12}N_6O_3$	289.12	Cream	50.00 (50.28)	4.20 (4.12)	29.15 (29.03)	-----
CuL_2	$C_{24}H_{22}N_{12}O_6Cu$	638.74	Green	46.73 (46.68)	4.02 (4.16)	28.45 (28.62)	1.96BM
$CoL_2(H_2O)_2$	$C_{24}H_{26}N_{12}O_8Co$	674.16	Reddish	45.09 (45.19)	3.65 (3.62)	28.08 (28.22)	5.18BM
NiL_2	$C_{24}H_{22}N_{12}O_6Ni$	633.18	Orange	48.29 (48.06)	3.69 (3.72)	28.13 (28.03)	diam.

6.2.1.1. IR spectra

In the IR spectra of the complexes (Table 6.2) the υN^1H (3433 cm^{-1}) and $\upsilon C{=}O$ (1665 cm^{-1}) stretching vibrations corresponding to the free ligand are not observed. This fact, along with the presence of a medium intensity band in 1495-1497 cm^{-1} range, assignable to $\upsilon C–O$, indicates that the deprotonated ligand is predominantly in the enolate form (B) in the complexes. On complex formation, the position of $\upsilon CH{=}N^2$ is shifted to the higher wave numbers, shift which indicates that the azomethine nitrogen atom is coordinated to the metal ion [26, 27]. This coordination behavior of the ligand is also proved by the appearance of IR bands due to $\upsilon M–O$ and $\upsilon M–N$ vibrations in the range 471–478 cm^{-1} and 428–450 cm^{-1}, respectively [28, 29]. The pyridine-ring-stretching, in-plane-ring-bending and out of-plane-ring-bending vibrations are found at 1502, 628 and 502 cm^{-1}, respectively. These absorptions are highly affected when nitrogen atom of pyridine ring takes part in coordination. In the complex (1) the position of these absorption bands is shifted to higher region. This indicates that the nitrogen of pyridine ring is involved in coordination. The above discussion reveals

that the ligand coordinates to the metal atom as a tridentate chelate. The complex $[Co(L)_2(H_2O)_2]$ **(2)** exhibit n(OH) and n(H_2O) bands in the 3124 and 681 cm^{-1} regions which are indicative of coordinated water in the complex [30, 31].

Table 6.2: IR bands and their assignments for ligand (L) and its Cu(II), Co(II) and Ni(II) complexes.

Comp.	υOH	υNH	υC=O	υCH=N	υC-O	υC=C	υPy	υC-Cl	υM-O	υM-N
LH	--	3433	1583	1697	--	1583	1502	772	--	--
1	--	--	--	1599	1497	1566	1534	742	478	445
2	3124, 681	--	--	1599	1495	1566	1533	736	473	428
3	--	--	--	1604	1496	1533	1461	743	471	450

6.2.1.2. Electronic spectra and magnetic studies

The electronic spectra of the ligand, shows two bands at 39,082 cm^{-1} and 29,832 cm^{-1} due to the benzene ring and to the π→π* transition of the chromophores (–C=N–NH–CO–), respectively. These bands shift to wave numbers in the electronic spectra of the complexes (38,750–37,025 cm^{-1} and 29,137–28,760 cm^{-1}, respectively). On the basis of electronic spectra, distorted octahedral geometry around Cu(II) ion in complex **(1)** is suggested [32]. The spectrum shows a low intensity broad band at 13,045 cm^{-1} (dx^2-y^2 →dxy transition) and a second band at 17,540 cm^{-1} which is assigned to dx^2-y^2→dxy,yz transition. This complex has magnetic moment 1.93 BM, which is higher than the spin-only value (1.70 BM) expected for one unpaired electron and offers possibility of an octahedral geometry [33]. Cobalt(II) complex **(2)** exhibits a typical electronic spectrum for octahedral species in the solid state [34, 35]. Two main bands are observed at 13,465 cm^{-1} and 18,295 cm^{-1}, which are assigned to the

[$^4A_{2g}(F) \leftarrow {}^4T_{1g}(F)$] ($\upsilon_2$) and [$^4T_{1g}(P) \leftarrow {}^4T_{1g}(F)$] ($\upsilon_3$) transitions. The room temperature magnetic moment value, 5.18 B.M., demonstrates that the Co(II) complex is paramagnetic and has a high-spin octahedral configuration with $^4T_{1g}(F)$ ground state [36]. Explanation of the magnetic behavior of Co (II) complexes is always difficult, due to the orbitally degenerate ground state of the ion when six-coordinate. The observed value of μBM at 298K corresponds to that for Co (II) (S = 3/2) centers with g = 2.48, the latter value indicating a significant orbital contribution. For the diamagnetic Ni(II) complex **(3)** the electronic spectra showed typical square-planar bands [34, 37] a medium intensity board band at 20,120 cm^{-1} and this may be regarded due $^1A_{2g} \leftarrow {}^1A_{1g}$ transition and a shoulder band at 23,875 cm^{-1} which may be attributed to $^1E_{1g} \leftarrow {}^1A_{1g}$.

6.2.1.3. Molar conductivity

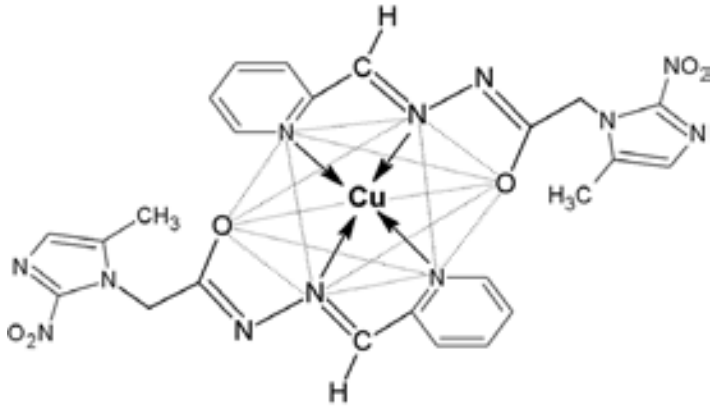
The molar conductivity (Λm) of 10^{-4} M solution of the complexes **(1-3)** in DMSO at room temperature was measured and all the complexes were found to be non-electrolytic in nature [38].

Cu- complex **(CuL_2)**	10 Ω^{-1} cm^2 mol^{-1}
Co- complex **($CoL_2(H_2O)_2$)**	16 Ω^{-1} cm^2 mol^{-1}
Ni- complex **(NiL_2)**	10 Ω^{-1} cm^2 mol^{-1}

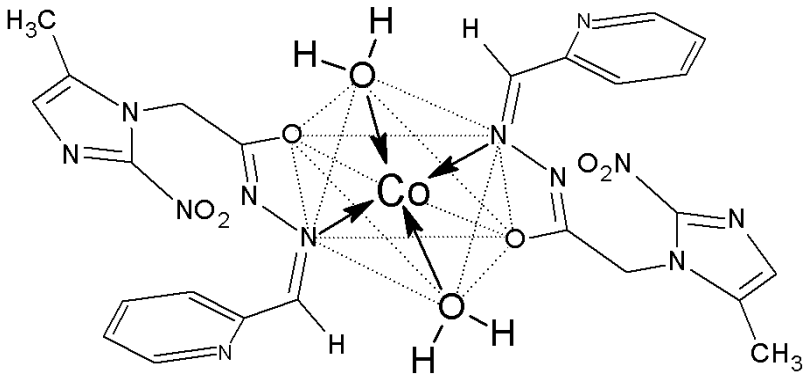
6.2.1.4. Thermogravimetric analysis

Thermal stability up to 260 ^oC for the complex (1) indicated lack of coordination water. Above this temperature thermal decomposition begins. For the complex (1), there is a single, exothermic process, between 280 and 650 ^oC, which corresponds to the loss of ligand. (5.26% of the sample weight). Organic part is lost in the 250–700 ^oC range and the experimentally obtained residue of CuO determining the percentage of 11.53% copper. The thermal decomposition of Co(II) complex (2) occurs in three

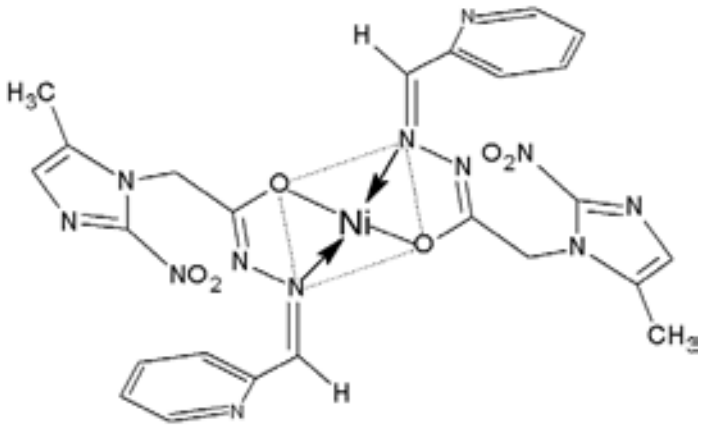
distinct stages. First, in 160–195^{o}C range corresponds to loss of two water coordinating molecules. The organic part is lost in two stages, with maximum at 380^{o}C and 500^{o}C, respectively, which corresponding to nitroimidazole fragment and the residue hydrazone, respectively. Ni(II) complex (3) is stable up to 280 ^{o}C and it decomposes in two cumulative stages, which corresponds of the loss of the ligand. Over 690^{o}C the thermogram registers a plateau region, which corresponding to NiO residue. Thus, based on these analytical and physico-chemical data, the proposed structures for the complexes are shown in Figure 6.7.



Cu L_2 complex (1)



$CoL_2(H_2O)_2$ Complex (2)



Ni L_2 Complex (3)

Figure 6.7: *Proposed structure of Cu(II), Co(II) and Ni(II) complexes.*

6.2.2. Nanosizing

The particle size and morphology was estimated from TEM micrographs. Figure 6.8 (a, b & c) shows the transmission electron microscopy (TEM) images of nanoparticles synthesized by reverse micro-emulsion technique. The typical histograms of size distribution for these metal complex nanoparticles (N1, N2 and N3), as shown in inset of Figure 6.8 (a, b & c), indicating the quality of spherical nanoparticles is very high in terms of size distribution. The digitized images were imported into the program, Image J and the populations of particles with respect to mean particle diameter were determined. The particles obtained by reverse micro-emulsion process resulted into the highly monodisperse, narrower and nearly spherical particle size distribution with reduced average diameter in the range of 15-25 nm as shown in Figure 6.8 (a, b & c). The nanosizing of these acyl-hydrazone ligand based metal complexes resulted in an increased solubility as shown by the formation of nanosuspensions in figure 6.9.

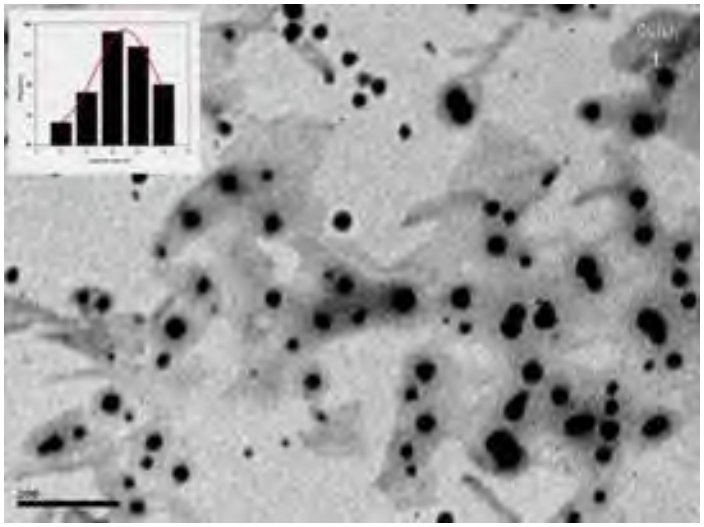


Figure 6.8 (a): TEM image of $Cu(L)_2$ complex (N1)

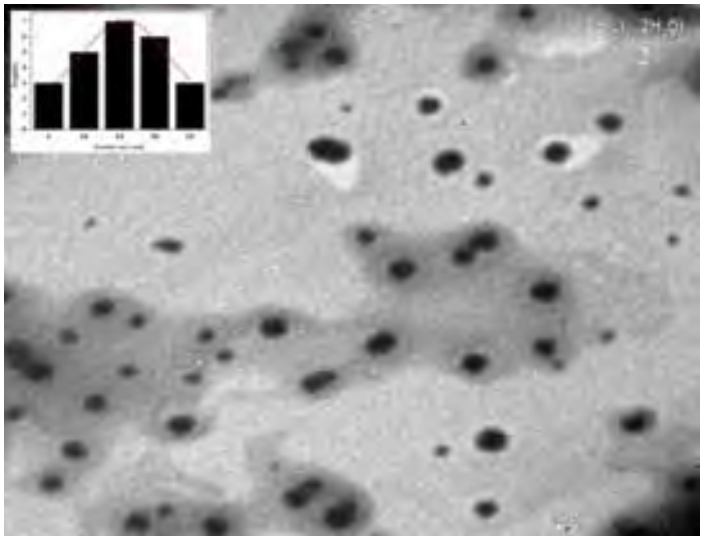


Figure 6.8 (b): TEM image of $Cu(L)_2.(H_2O)_2$ complex (N2)

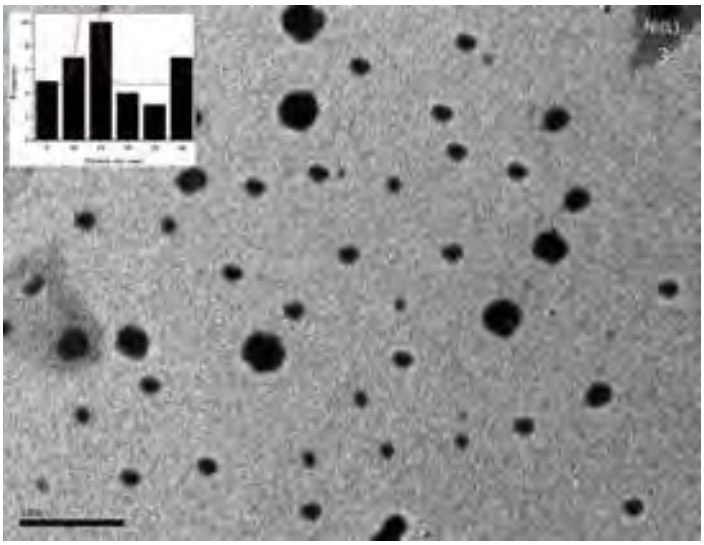


Figure 6.8 (c): TEM image of $Ni(L)_2$ complex (N3)

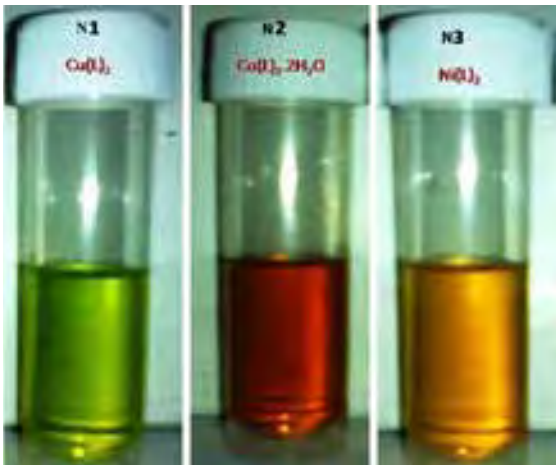


Figure 6.9: Nanosuspensions of Cu(II), Co(II) and Ni(II) complexes (N1, N2 and N3 respectively). *Nanoparticles were synthesized by reverse micro-emulsion technique.*

6.2.2.1. *In vitro* antiamoebic activity

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of the ligand (L), its metal complexes (CuL_2, CoL_2, NiL_2) and their nanosized particles (N1, N2, and N3 respectively) by microdilution method using *HM1: IMSS* strain of *Entamoeba histolytica*. The results are summarized in Table 6.3. The data is presented in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with the most widely used antiamoebic medication metronidazole with 50% inhibitory concentration (IC_{50}) of 1.80 µM in our experiments. Although the investigation of these complexes was limited by synthetic considerations and only three complexes were prepared. The study was however interesting because the nanosized particles of these complexes were synthesized to check their probable antiamoebic efficacy. The ligand, its Cu(II), Co(II) & Ni(II) complexes and their nano sized particles (N1, N2 & N3 respectively) showed an interesting inhibitory pattern. The ligand showed IC_{50} value of 2.65 µM. The complexes showed improved potency as compared to the metal free ligand. $Cu(L)_2$ complex showed increased activity (IC_{50} = 2.03 µM) than the ligand which indicates that the insertion of metal increases the activity. The replacement of Cu by Co led to an increase in the potency (IC_{50} = 0.89 µM), which is two times higher than the reference drug metronidazole (IC_{50} = 1.80 µM). The further replacement of Co by Ni resulted in a decrease in activity (IC_{50} = 2.18 µM). A similar pattern of inhibitory behavior was followed by the nanosized particles (N1, N2 & N3), with a slight increase in their activity compared to their parent complexes. N2 corresponding to $Co(L)_2.2H_2O$, showed a slight increase in activity(IC_{50} = 0.86 µM), followed by N1 and N3 (IC_{50} = 1.98 µM & 2.10 µM respectively). From the results it is however

evident that the modification of the synthesized complexes into nanosized particles did not bring any appreciable change in their activity. Despite of the fact that the solubility of these nanosized particles in water got increased it did not help them to attain expected antiamoebic activity, which gives an indication that some other factors besides the solubility are also responsible for the activity of a compound. From the results of antiamoebic activity it can be concluded that the $Co(L)_2.2H_2O$ metal complex is an excellent *E. histolytica* inhibitor with its nanosized particles (N2), having comparable or slightly enhanced activity followed by N1 > $Cu(L_2)$ > N3 > $Ni(L)_2$ > Ligand (L).

The results were also statistically evaluated by analysis of variance. The null hypothesis was tested using t-test. The significativity of the difference between the IC_{50} values of metronidazole and the compound CoL_2 and N2 was evaluated by t-test. The values of the calculated T were found higher than the Table value of T at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

Table 6.3: *In vitro* antiamoebic activity of the ligand (L), its metal complexes (CuL_2, CoL_2 and NiL_2) and their nanosized particles (N1, N2, and N3 respectively) against *HM1: IMSS* strain of *Entamoeba histolytica* and toxicity profile.

Compound	Antiamoebic activity		Toxicity Profile	
	IC_{50} (µM)	**S.D[a]. (±)**	**IC_{50} (µM)**	**Safety Index (SI)**
Ligand (L)	2.65	0.16	>100	>37.73
CuL_2	2.03	0.18	93.2	45.91
CoL_2	**0.89**	0.22	>100	**>112.36**
NiL_2	2.18	0.24	63.8	29.26
N1	1.98	0.22	90.8	45.85
N2	**0.86**	0.20	96.8	112.55
N3	2.10	0.24	84.7	47.05
MNZ	1.80	0.18	>100	>55.55

Compounds with bold font (IC_{50} value) are more active than metronidazole (MNZ).

6.2.2.2. *In vitro* cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [39, 40] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [41-43].

In the present study, some newly synthesized compounds were screened for their antiamoebic activity and then evaluated for their cytotoxicity against *Human hepatocellular carcinoma cell line (*HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A sub-confluent population of HepG2

cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13-100 μM. The cell viability (%) obtained with continuous exposure for 48 h are depicted in Figure 6.10. The cytotoxicity of all the compounds was found to be concentration-dependent. Figure 6.10 depicts that all the test compounds including the reference compound metronidazole showed 100% viability, at the concentration range of 3.13 μM and up to a concentration of 25 μM all the compounds showed a viability of ≥76%. On increasing the concentration range up to 50 and 100 μM the percentage viability began to decrease which indicates the increase in toxicity of the test compounds. Co(II) complex (CoL_2) showed comparatively less toxicity than Ni(II) and Cu(II) complexes, however it was higher than the Ligand (L). The results indicate that the insertion of metal ion in the coordination sphere increases the toxicity. This was also true for their nanosized particles (N1, N2 and N3), which showed slightly higher toxicity than their parent complexes tested. Based on the results of antiamoebic activity and cytotoxicity studies it can concluded that the coordination of metal with the ligand increases both the antiamoebic activity and cytotoxicity, but the concentration at which the complexes inhibit the growth of *E. histolytica* is almost 50-100 times lower than the concentration at which it shows toxicity. From this investigation it was also observed that the modification of metal complexes into nanosized particles slightly enhances both their antiamoebic activity and toxicity which can be attributed to their increased solubility. The results of toxicity profile of all the tested compounds are given in Figure 6.10. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Table 6.3.

Compound **CoL_2** showed higher safety index values, better than metronidazole. From the results of antiamoebic activity and cytotoxicity it can be inferred that all the compound $CoL_2.(H_2O)_2$ and its nanosized particles (N2) are excellent *E. histolytica* inhibitors. These results also showed that the compound **CoL_2** and **N2** despite of being highly antiamoebic do not show any marked toxicity on human cell line and have safety index values of >112.36 and 112.55 which is better than metronidazole (>55.55).

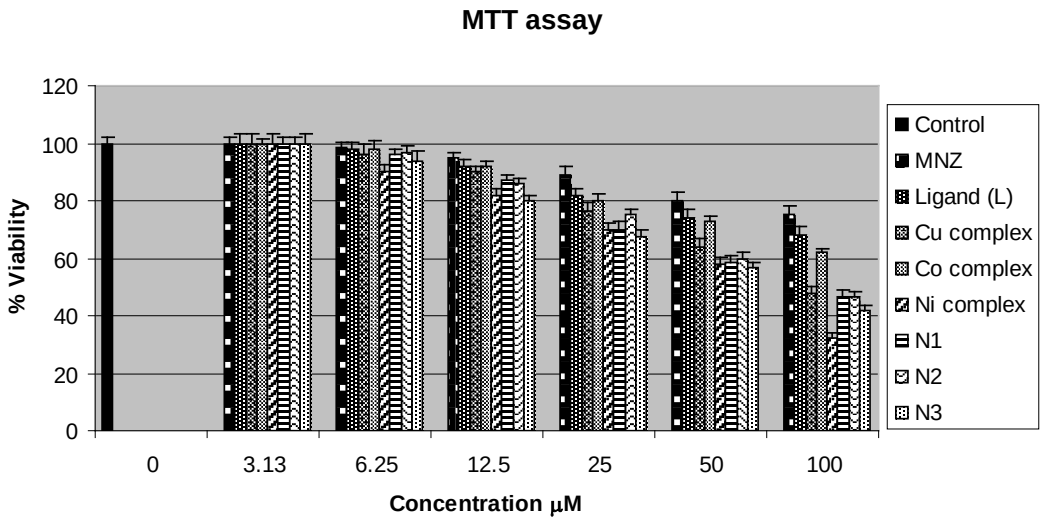


Figure 6.10: Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with Metronidazole, ligand (L), its Cu, Co, Ni(II) metal complexes and their nanosized particles N1, N2 & N3 evaluated by MTT assay.

6.3. EXPERIMENTAL

6.3.1. Synthesis

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results are uncorrected. Elemental analyses (C, H, N) was performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ± 0.4% of the theoretical values. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-d_6/ $CDCl_3$ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Thermogravimetric analysis of the complexes was performed on a thermogravimetric analyzer (Perkin-Elmer) under nitrogen atmosphere with the heating rate of 10^{o}C/min from 50^{o}C to 900^{o}C. Room temperature magnetic susceptibility was measured at 298 K by a Vibrating sample Magnetometer 155, E-112 ESR Spectrometer, Varian, USA using nickel as standard and such values were expressed as effective magnetic moment (μ_{eff}) in Bohr Magneton (B.M.). Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapor staining.

6.3.1.1. Synthesis of the ligand (L)

2-(5-methyl-2-nitro-1H-imidazol-1-yl)acetohydrazide

2-(5-methyl-2-nitro-1H-imidazol-1-yl)acetohydrazide was prepared from 5-methyl-2-nitro-1*H*-imidazole by a reported method [44].

***2-(5-methyl-2-nitro-1H-imidazol-1-yl)-N'-[(E)-pyridin-2-ylmethylidene]aceto hydrazide* (L)**

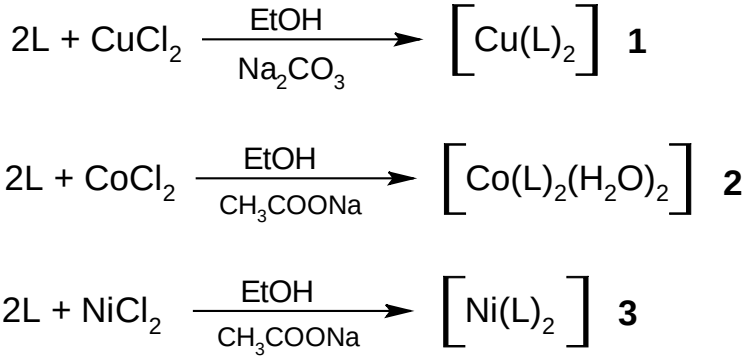
2-Pyridinecarbaldehyde (10 mmol) was added over a solution consisting of *2-(5-methyl-2-nitro-1H-imidazol-1-yl)acetohydrazide* (10 mmol) in 20 ml ethanol and the reaction mixture was kept under reflux for about 3 h. The solvent was removed under reduced pressure and the microcrystalline mass obtained was recrystallised from methanol (mp. 128-130°C).

Cream; Yield: 89.3%; Anal. Calc. For $C_{12}H_{12}N_6O_3$: C 50.00, H 4.20, N 29.15%; found: C 50.28, H 4.12, N 29.03%; IR $\nu_{max}cm^{-1}$: 3433 (NH), 3142 (C-H stretch), 1697 (C=O), 1583 (C=N), 1537, 1390 (-NO_2); ^{1}H NMR (DMSO-d_6) δ(ppm): 12.08 (s,1H, NH, hydrazone), 8.64 (d, 1H, *J* = 4.6 Hz, py ring), 8.11 (s, 1H, imidazole ring), 8.03-7.85 (m, 2H, py ring), 7.46 (t, 1H, *J*= 7.8 Hz, py ring), 5.44 (s, 1H, -N=CH-), 3.38 (s, 2H, methylenic), 2.33 (s, 3H, methyl); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.9 (C=O), 164.0 (C=N), 149.5, 136.4, 130.5, 129.1, 128.5, 127.0, 125.0, 25.5 (CH_2), 12.6 (CH_3); ESI-MS m/z: $[M+1]^+$ = 289.10

6.3.1.2. Synthesis of the complexes

We prepared the complexes with $CuCl_2$, $CoCl_2.6H_2O$ and $NiCl_2.6H_2O$ salts. The ethanolic solution of metallic ion salt (1 mmol/5 mL ethanol) was mixed with stirring with a hot clear ethanolic solution of the ligand (L) (2 mmol/20 mL ethanol). After refluxing the solution for 2 h. Na_2CO_3 or CH_3COONa was added until the pH = 8–8.5 and after cooling the contents, colored complex separated out in each case. It was

filtered, washed successively with water, hot ethanol, cold ethanol and diethylether and finally dried under vacuum.



Scheme 6.2: Synthesis of complexes from Ligand (L)

6.3.1.3. Synthesis of nanoparticles

The synthesis of nanoparticles was carried out in microemulsions by direct precipitation of the active substance in aqueous cores. The microemulsion system used to synthesize the nanoparticles was CTAB/n-Hexanol/water. Distilled water is used. The surfactant is CTAB (Spectrochem, India); The oil phase is formed by n-Hexanol (min 98%, Sigma Chemical Co.). To prepare the nanoparticles, the synthesized complexes (CoL_2, $CuL_2(H_2O)_2$ and NiL_2) were used as active components respectively and solubilized in chloroform (99%, stabilized with 0.75% of methanol, Janssen Chemica). The nanoparticles are observed by transmission electron microscopy using a Philipps EM301 microscope.

The preparation of the nanoparticles consists of several stages. First, the empty microemulsion is prepared and then the active compound soluble in chloroform is added dropwise. Different values of factor R = [water]/[E170] have been used. This ratio is in general proportional to the diameter of the water droplet. The preparation of the empty microemulsion is thus stage 1 where the organic phase (n-Hexanol) is added to CTAB with a syringe and stirred until the complete dissolution of the

surfactant. The added volumes depend on the composition of the microemulsion (and thus on the value of R). Stage 2 consists of the addition of the amount of water determined by the R factor and the microemulsion is stirred until the solution becomes transparent. The so-prepared microemulsion is stable. In stage 3, the active compound in chloroform is injected dropwise into the microemulsion under magnetic stirring (0.4 ml in all experiments). The final treatment under ultrasound or magnetic stirring occurs during 15 min. The nanoparticles are obtained after washing with distilled water and repeated centrifugation.

6.3.2. *In vitro* antiamoebic assay

All the test compounds; ligand (L), its metal complexes (CuL_2, $CoL_2.2H_2O$, NiL_2) and their nanosized particles (N1, N2, and N3 respectively) were screened *in vitro* for antiamoebic activity against *HM1:IMSS* strain of *Entamoeba histolytica* by microdilution method [45]. The detailed procedure of this assay is given in Chapter 2.

6.3.3. Cytotoxicity studies (MTT assay)

6.3.3.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37 ^0C in a saturated humidity atmosphere containing 95% air/5% CO_2 [46]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

6.3.3.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [47]. The detailed procedure of this assay is given in Chapter 2.

6.4. CONCLUSION

This study achieved the synthesis of acylhydrazone ligand based Cu(II), Co(II) and Ni(II) transition metal complexes and their nanosized particles using reverse microemulsion technique. The results of antiamoebic screening revealed that the $Co(L)_2.2H_2O$ metal complex is an excellent *E. histolytica* inhibitor with its nanosized particles (N2), having comparable or slightly enhanced activity followed by N1 > $Cu(L_2)$ > N3 > $Ni(L)_2$ > Ligand (L). The cytotoxicity of the screened compounds was found to be concentration dependent. Based on the results of antiamoebic activity and cytotoxicity studies it can concluded that the coordination of metal with the ligand increases both the antiamoebic activity and cytotoxicity, but the concentration at which the complexes inhibit the growth of *E. histolytica* is almost 50-100 times lower than the concentration at which it shows toxicity. From this investigation it was also observed that the modification of metal complexes into nanosized particles slightly enhances both their antiamoebic activity and toxicity which can be attributed to their increased solubility.

6.5. REFERENCES

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