Pharmacological studies on 1,4-disubstituted-1,2,3-triazoles

By

Yasmeen Niazi

In

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Department of Biochemistry

Faculty of Biological Sciences

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A thesis submitted in the partial fulfilment of the requirement for the degree of Master of Philosophy

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Supervised by

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Islamabad-Pakistan
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CERTIFICATE

This thesis, submitted by **Ms. Yasmeen Niazi** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor: Dr. Bushra Mirza

External Examinar: Dr. Rani Faryal

Chairman: Dr. Salman Akbar Malik

Dated: August 10, 2010

DEDICATED

TO

My Loving

Father

Who always stood behind me and knew I would succeed

Contents

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*All praises to **Allah Almighty**, the most beneficent, the most merciful, Who gave me strength and enabled me to undertake and execute this research task. Countless salutations upon the **Holy Prophet Hazrat Muhammad** (Sallallaho Allaihe Waalahe Wassalum), the city of knowledge for enlightening with the essence of faith in Allah and guiding the mankind, the true path of life.*

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Yasmeen Niazi

LIST OF ABBREVIATIONS

DNA	Deoxyribonucliec acid
cm	Centimeter
CFU	Colony Forming Unit
DMSO	Dimethylsulphoxide
gm	Gram
g/L	Gram per Litre
g/ml	Gram per Millilitre
mg/ml	Milligram per Millilitre
µg/ml	Microgram per Millilitre
$FeSO_4$	Ferrous Sulphate
H_2O_2	Hydrogen peroxide
pBR322	Plasmid BR322
KI	Potassium Iodide
I	Iodine
M	Molar
mm	Millimetres
mM	Milli molar
ppm	Parts per million
TBE	Tris Boric Acid EDTA
UV	Ultra violet
OC	Open circular
SC	Super coiled
IC_{50}	50% inhibitory concentration

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ABSTRACT

The present study aimed at screening a set of newly synthesized 1,4-disubstituted-1,2,3 triazoles for their possible biological activities including antimicrobial, antitumor, antioxidant and cytotoxic activities. These newly synthesized 1,2,3 triazoles included 15 compounds with position 1 of the central triazole ring containing phenyl and 1-hydroxycyclohexyl groups and position 4 containing alkyl, phenyl, and substituted aryl groups. All of these compounds were subjected to antibacterial assay using agar well diffusion method for determining zones of inhibition and minimum inhibitory concentrations (MIC). The assay was done against five bacterial strains including three gram negative (*Brodetella bronchiseptica*, *Escherichia coli* and *Enterobacter aerogenes*) and two gram positive strains (*Staphylococcus aureus* and *Micrococcus luteus*). Only compound CC17 showed some antibacterial activity with MIC values ranging from 0.2 to 0.8 against different strains, however, it did not give any activity against *E.coli* bacteria. The antifungal activity of these compounds was tested by agar tube dilution method. Compound CC17 gave highly significant results and showed 100% inhibition against *A. flavus*, 80% inhibition against *F. solani* and more than 60% inhibition against *A. niger* and *A. fumigatus*. Cytotoxic analysis of compounds was done using Brine shrimp lethality assay some of the compounds proved highly toxic against shrimp larvae e.g. CC6, CC8, CC16 and CC17. Antitumor potato disc assay was carried out to examine antitumor activity of the compounds at four different concentrations of all 15 compounds. Highest tumor inhibition was exhibited by compound CC16. Antioxidant and prooxidant nature of the compounds was studied by free radical induced DNA damage analysis. Some compounds like CC1 and CC8 gave DNA protective activity at 3000 ppm concentration. Others were protective against free radical damage at lower concentrations, however, the extent of protective activity against radical damage was not very pronounced.

Data obtained from these tests indicate potential of these compounds as biological agents and also enabled us to compare their antimicrobial, antifungal and antitumor properties.

INTRODUCTION AND LITRATURE SURVEY

1.1. Azoles:

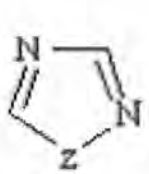
Azoles are five membered heterocyclic rings containing at least one nitrogen atom in their ring structure like Isoxazole, Thiazole, Pyrazole and **Triazole** (Joule and Mills 1995).

1.2. Triazoles:

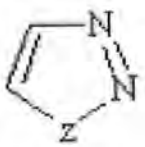
The **triazoles** consist of two isomers having the formula $C_2H_3N_3$. These are aromatic ring compounds similar to the azoles pyrazole and imidazole, but with an additional nitrogen atom in the ring structure. Triazoles are used in many antifungal drugs and fungicides, and the triazole-based drugs are more selective for fungi than mammalian cells than the azole-based antifungal compounds. Synonyms for both of these triazoles sometimes denote that a proton is attached in the 1-position, as for example, the naming 1H-1,2,3-triazole or 1,2,3-1H-triazole.

1.2.1 Constitution of Triazoles:

In the figure 1.1 two types of triazoles are shown:



(a)



(b) **(Z=NH)**

Fig.1.1: structure of (a) 1,2,4 triazole (b) 1,2,3 triazole

Triazole ring derivatization is based on the phenomenon of bioisosterism in which replacement of oxygen of oxadiazole nucleus with nitrogen atom yields triazole analogue (Kartritzky, 1985).

1.2.2. 1,2,3-Triazoles:

1,2,3-Triazole is one of a pair of isomeric triazoles, which have a five-membered ring of two carbon atoms and three nitrogen atoms. 1,2,3-Triazole is a basic aromatic heterocycle (Gilchrist, 1992).

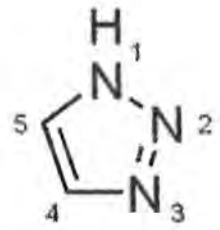


Fig.1.2. 1,2,3 triazole position numbers in the ring

1.2.3. 1,2, 4-Triazoles:

1,2,4-Triazole is the other member of the isomeric triazoles, which also have the same molecular formula as 1,2,3 triazole but differ form them in the position of one of the nitrogen atoms. 1,2,4-Triazoles are basic aromatic heterocycles. 1,2,4-Triazole derivatives find use in a wide variety of applications, most notably as antifungals such as fluconazole and itraconazole.1,2,4-Triazoles can be prepared by the Einhorn-Brunner reaction or the Pellizzari reaction (Potts, 1961).

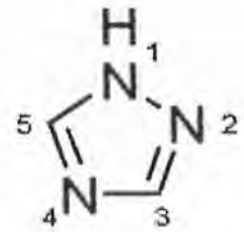


Fig.1.3. 1,2,4 triazole position numbers in the ring

1.2.4. Synthesis of 1,2,3 triazoles:

There are different methods of preparing these compounds. In general they are prepared through the coupling reaction between alkynes and azides at high temperature to form a mixture of 1,4-substituted- and 1,5-substituted-1,2,3triazoles (Huisgen, 1984; Padwa, 1991; Zhao *et al.*, 2006). Investigations in this area led to the 'click' chemistry approach (Kolb *et al.*, 2001; Rostovtsev *et al.*, 2002). Another novel alternative is the copper (I)-catalyzed Huisgen cycloaddition reaction of azides and terminal alkynes which has received much attention since its discovery. The methodology has found applications in drug discovery and bioconjugations (Lewis *et al.*, 2002; Rheingold *et al.*, 2000; Tornøe *et al.*, 2002; Wang and Lin, 2003; Trofimenko *et al.*, 2003; Krasinski *et al.*, 2004). Exclusive regioselectivity, wide substrate scope and mild reaction conditions have made it the method of choice for making permanent connections by means of 1,4-disubstituted 1,2,3-triazoles(Zhao *et al.*, 2006).

1.2.5. Stability of 1,2,3 triazoles:

It is a surprisingly stable structure compared to other organic compounds with three adjacent nitrogen atoms. However, flash vacuum pyrolysis at 500 °C leads to loss of molecular nitrogen (N_2) to produce aziridine. Certain triazoles are relatively easy to cleave due to so-called ring-chain tautomerism. One manifestation is found in the Dimroth rearrangement (Gilchrist, 1992).

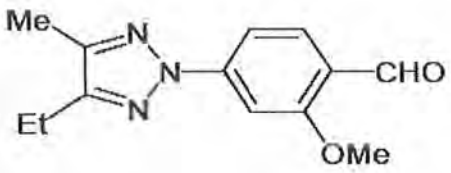
1.2.6. Industrial importance of 1,2,3 triazoles:

1,2,3-Triazoles have a large number of industrial applications in agrochemicals, corrosion inhibitions, dyes, optical brighteners and they are also biologically active agents (Wamhoff, 1984; Abu-Orabi *et al.*, 1989).

Because of their biological and chemical importance, greatest attention has been paid to the synthesis of 1,2,3-triazoles condensed with other heterocycles and investigation of their biological activity in medicinal chemistry. Even in 1935 research was started on the possibility of using 1,2,3-triazolo-pyrimidines (8-azapurines) as chemotherapeutic agents for the treatment of various diseases and particularly malignant tumors (Benson and Savell, 1950; Boyer, 1965) .

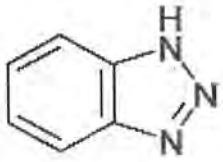
The search for new biologically active compounds in the series of condensed 1,2,3-triazoles is continuing to the present day. Thus, for example, compounds inhibiting benzodiazepine and adenosine receptors (Betti *et al.,* 1998; Biagi *et al.,* 2002) and substances acting against the hepatitis C virus (Wang *et al.,* 2005) were found.

In contrast to other nitrogen heterocycles such as pyrroles, imidazoles, pyridines, and pyrazines, the pyridazines and 1,2,3-triazoles have seldom been found in nature as constituents of natural products. Some of their derivatives that have found diverse uses in synthetic, analytical, medicinal, pharmaceutical, agrochemical, and photographic chemistry, and in other applications as corrosion inhibitors, photostabilizers, dyestuffs and fluorescent whiteners, and asymmetric dihydroxylation catalysts are shown in the **fig.1.4.(a-d).** (Tišler and Stanovnik 1968; Tisler and stanovnik 1979; Tišler and Stanovnik 1984; Tišler and Stanovnik 1990; Coates, 1996 ;Katritzky and Rees, 1996; Scriven 1996; Stanovnik 1997; Weissberger and Taylor 1980; Kadaba *et al.,* 1984 ; Kadaba, 1984; Albert 1986; Wamhoff *et al.,* 1984; Fan and Katritzky 1996)

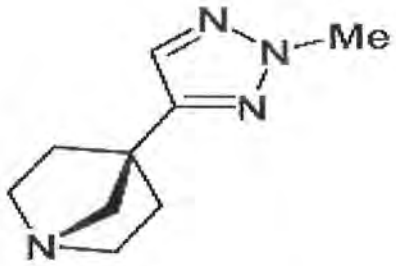


(a) Fluorescent whitening agent

(b) Photostablizer



(c) 1*H*-Benzotriazole
(Synthetic auxiliary)



(d) Muscarinic receptor ligand

Fig.1.4. (a-d) some industrially used 1,2,3 Triazoles

1-methyl-6-dimethylaminobenzotriazole (Miglaresje, 1944) and 2-p-nitrophenylbenao[h]triazolelf]quinoline (Farbenindusat, 1938) are claimed, respectively, as a sunburn preventive and for inclusion in cellophane films because of their ability to absorb ultraviolet light. The sodium salt of ethyl 5-hydroxy-1-sulfanilyl-v-triazole-4-carboxylawtaes was found to be approximately as effective a bacteriostatic agent as sulfapyridine toward *E. coli* as well as toward pneumococci, meningococci, and gonococci *in vitro,* but was inferior to sulfathiazole (Benson and Savell 1950).

Several triazolopyrimidines are structural analogs of various purines (Roblin *et al.,* 1945). All of these triazolopyrimidines were found to have some growth-inhibiting action on *E. coli* and *S. aureus*. The guanine analog, 5-amino-7-hydroxy-v-triazolo [d] pyrimidine when used in combination with sulfanilamide, exerted a synergistic bacteriostatic effect toward *S. aureus* (Roblin *et al.,* 1945).

1.3. Heterocyclic Compounds and Drug discovery:

New healing materials and remedies have been discovered by human beings since ages, which was possible only by observing life in nature. A large number of pharmaceutical products are mimics of natural products with biological activity, which include many heterocycles. Some of the most significant advances have been in the fight against disease, by designing and testing new structures, many of these new structures are heteroaromatic derivatives. Similarly many pesticides, antibiotics such as penicillins and cephalosporins, alkaloids such as vinblastine, ellipticine, morphine, and reserpine, and cardiac glycosides such as the class of digitalis are heterocyclic natural products of significance for human and animal health. Pharmaceuticals for a better living have been designed and produced by researchers after drawing their inspiration from natural products. In the same light, pesticides, insecticides, and weed killers followed natural models, and a significant part of such biologically active compounds are heterocycles.

New discoveries and modernization led to other important practical applications of heterocycles, for example dyestuffs, copolymers, solvents, photographic sensitizers and developers, and in the rubber industry antioxidants and vulcanization accelerators (Pozharskii *et al.*, 1997).

1.4. BIOASSAY:

Bioassay is a method for the estimation of the effects that result in a biological system after it is exposed to a substance. This is done by comparing the activity of living organisms and/or their parts under standardised conditions versus the conditions under investigation.

The **biological material** in which the effect is measured can be subcellular components, microorganisms or groups of animals. Bioassays are mostly used in situations where there are a number of steps between the substance exposure and the behaviour observed which are not understood very well or when the substance is a complex mixture of materials and it is not clear what the active components are for example a plant extract. Bioassays that analyse the effects of an exposure, model the effect of a substance in the real world. Complex biological responses can be estimated

by laboratory culture tests which use, for example, bacteria or cells cultured in a petri dish, by tissue or organ culture, which isolates pieces of tissue, or whole organs in a petri dish, or in animals. Tests in which direct effects on an organism are measured are frequently easier to extrapolate to real world situations than more indirect tests. (http://www.iscid.org/encyclopedia/Measure)

Bioassays can be categorized as qualitative and quantitative.

Qualitative bioassays:

Qualitative bioassays are used for assessing the physical effects of a substance that may not be quantified, such as abnormal development or deformity.

Quantitative bioassays:

Quantitative bioassays involve estimation of the concentration or potency of a substance by measurement of the biological response that it produces. Quantitative bioassays are typically analyzed using the methods of biostatistics. (http://en.wikipedia.org/wiki/bioassay).

1.5.1 Antimicrobial assay:

Drug resistance to antibiotics, especially the multiple drug resistance (MDR), is nowadays an alarming and major public health concern worldwide (Mitscher *et al.*, 2006).

Some antibiotics have become out dated and ineffective because of drug resistance (Ekpendu *et al.*, 1994). Many strains of bacteria have developed resistance through the years following the clinical use of the first antibiotics and there is continuous emergence and spreading of antibiotic-resistant bacteria (Lim and Webb, 2005). A great variety of human, animal and plant diseases are caused by pathogenic microbes (fungi, bacteria and algae). Bacterial and fungal infections have been a major cause of death in plants and animals (Rehman *et al.*, 2001)

During last three years many new antibiotics have been produced by the pharmacological but microorganisms has developed resistance to many of these drugs (Cohen, 1992). Thus there is still a need for seeking new molecules that can be added to the existing collection of drugs in order to treat these infections (Fluit *et al.*, 2006).

can be added to the existing collection of drugs in order to treat these infections (Fluit *et al.*, 2006).

The bacterial and fungal strains used in this study were selected on the basis of their importance as opportunistic human pathogen.

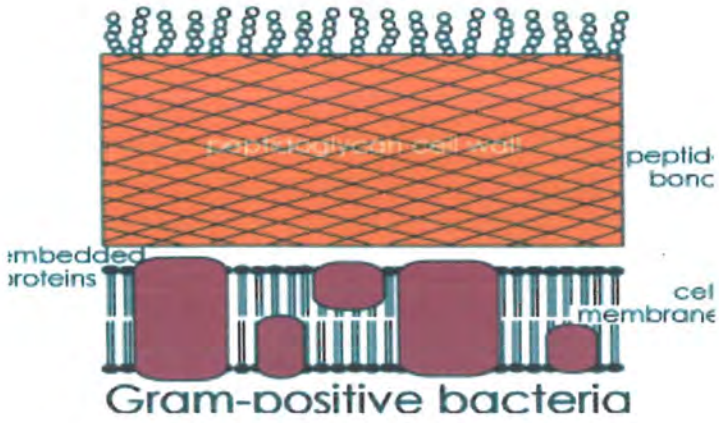
1.5.2 Antibacterial Assay:

Different methods are being used to detect the antimicrobial activity of compounds. Agar well diffusion method is one of the usual methods used to test the antibacterial activity. Bacterial strains used in this study are Staphylococcus aureus, *Micrococcus luteus*, *Brodetella bronchiseptica* , *Escherichia coli, and Enterobacter aerogenes.*

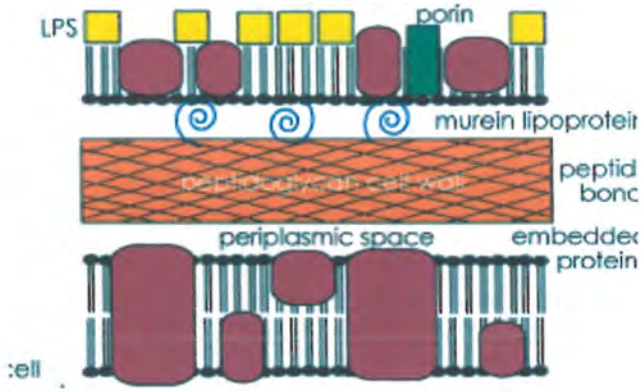
1.5.2.1 Gram positive and gram negative bacteria:

Bacteria can be differentiated into two types i.e. Gram positive or Gram negative by testing them with the "Gram stain". The stain attaches to part of the bacterial cell wall called peptidoglycan and causes a purple tinge. Because gram-positive bacteria have more layers of peptidoglycan in their cell walls than gram-negative, they can retain the dye.

Both gram-positive and gram-negative bacteria contain a cell wall made up of peptidoglycan and a phospholipid bilayer with membrane-spanning proteins. The existence of an outer membrane and the presence of only few peptidoglycan layers in the cell wall differentiate Gram-negative bacteria from Gram-positive ones **(Fig.1.5)**. The peptidoglycan is situated in the periplasm, a gap packed with fluid sandwiched between the plasma membrane and the outer membrane. Unlike Gram-positive cell walls, there is no teichoic acid in the Gram-negative cell walls. In addition, the cell walls of Gram-negative bacteria are more prone to mechanical breakage because of the low amount of peptidoglycan (Shagam, 2006; Wheelis, 2007).



(a) Gram positive bacterial cell wall



(b) Gram negative bacterial cell wall

(http://www.hhmi.org/biointeractive/Antibiotics_Attack/bb_2.html).

Fig.1.5. (a) cell wall of gram positive bacteria (b) cell wall of gram negative bacteria

Enterobacter aerogene is a Gram-negative, rod-shaped nosocomial and pathogenic bacterium that causes opportunistic infections including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections (Prescott *et al.*, 2002; Kerr *et al.*, 2003). It is responsible for food spoilage and causes infections in immunocompromised hosts (Selma *et al.*, 2003).

Staphylococcus aureus is a facultatively anaerobic, gram positive coccus and is the most common cause of *staphylococcus* infections. It is frequent part of the skin flora found in the nose and on skin. About 20% of the human population are long-term carriers of *S. aureus* (Kluytmans *et al.*, 1997). It appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies (Ryan and Ray 2004). *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), chest pain, bacteremia and sepsis (Ogston, 1984).

S.aureus bacteria growing under anaerobic conditions produce toxic shock syndrome. It is characterized by the sudden arrival of high fever, vomiting, diarrhea, and muscle aches, followed by low blood pressure (hypotension), which can lead to shock and cause death. There may be a skin rash similar to sunburn, with flaking of skin (Ala'aldeen and Hiramatsu 2004).

Escherichia coli is a Gram negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some can cause serious food poisoning in humans, and are occasionally responsible for product recalls (Vogt and Dippold 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, (Bentley and Meganathan 1982) and by preventing the establishment of pathogenic bacteria within the intestine (Hudault *et al.*, 2001; Reid *et al.*, 2001). Virulent strains of *E. coli* can cause stomach and intestinal inflammation, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible

for hemolytic-uremic syndrome (HUS), inflammation of abdomen lining, breast inflammation, septicemia and Gram-negative pneumonia (Todar, 2007).

Micrococcus luteus *are* Gram-positive cocci that are 0.5 to 3.5 micrometers in diameter and usually arranged in tetrads or irregular clusters. *M. luteus* can be found in many places such as the human skin, water, dust, and soil. Micrococcus is generally thought of as harmless bacterium, but there have been rare cases of Micrococcus infections in people with compromised immune systems, as in HIV patients (Smith *et al.*, 1999). The bacteria can degrade compounds in sweat, and on the skin, into ones producing unpleasant odours (foot odor, for instance). Rarely, it may also be associated with infections in the blood (sepsis), or endocarditis - an infection of the lining of the heart. Individuals with such infections often have a weakened immune system. *M. luteus* is not considered pathogen, or "disease-causing" organism of healthy people (Hug *et al.*, 1999).

Bordetella bronchiseptica: is a small, gram-negative, rod-shaped bacterium of the genus *Bordetella* (Hewlett, 1995). It is a respiratory tract pathogen of mammals (Ferry, 1910). *B.bronchiseptica* is an uncommon cause of bacterial infection in humans, but can cause significant disease, particularly among those who are immunocompromised and have exposure to dogs.In dogs, it causes infectious tracheobronchitis or "kennel cough." It is believed to be the genetic ancestor to *B. pertussis*, the organism causing "whooping cough" (Parkhill, 2003).

1.5.3. Antifungal activity:

Life threatening infections caused by pathogenic fungi are becoming increasingly common, especially in those individuals with suppressed immune systems such as cancer patients and patients with AIDS (Georgopapadakou and Walsh 1996).
Pathogenic fungi can be characterized as either primary or opportunistic pathogens. Primary pathogens are those that have the ability of causing disease in otherwise healthy hosts, whereas opportunistic pathogens are those that generally require evident immunosuppression in order to cause disease. Primary fungal pathogens can also infect immunocompromised hosts as well (Fish *et al.*, 1990 ; Ampel *et al.*, 1993).

Pathogenic fungi can be characterized as either primary or opportunistic pathogens. Primary pathogens are those that have the ability of causing disease in otherwise healthy hosts, whereas opportunistic pathogens are those that generally require evident immunosuppression in order to cause disease. Primary fungal pathogens can also infect immunocompromised hosts as well (Fish *et al.*, 1990 ; Ampel *et al.*, 1993). Antifungal antibiotics are a significant group of drugs and have an important role in the control of fungal diseases. The need for novel, safe and more effective antifungal compounds are a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host (Dhanasekaran *et al.*, 2008).

In the present study compounds are tested against following pathogenic fungi.

Aspergillus flavus is a common mold in the environment, and can cause problems in stored grains. It can also be a human pathogen, linked to the aspergillosis of the lungs and sometimes causing corneal, otomycotic, and nasoorbital infections. Many strains produce large quantities of aflatoxin (Klich, 2007), which is one of the etiological agents for hepatocellular carcinoma (Crawford, 2005). *Aspergillus flavus* is the second important reason of invasive and non-invasive aspergillosis (Hedayati *et al.*, 2007). In addition, it is the main Aspergillus species infecting insects, and it is also able to cause diseases in economically important crops, such as maize and peanuts, and to produce strong mycotoxins (Pasqualotto, 2008).

Cutaneous aspergillosis, wound infections and osteomyelitis following trauma, keratitis and Chronic granulomatous sinusitis are among the most common problems associated with *A. flavus* (Hedayati *et al.*, 2007). At least 80% of Aspergillus keratitis cases are associated with *A. flavus* (Khairallah *et al.*, 1992).

A. flavus produce many secondary metabolites among them the most important are aflatoxins which are the most toxic and strong carcinogenic natural compounds ever characterized (Hedayati *et al.*, 2007). Aflatoxin can infect crops before harvest or during storage, putting humans and other mammals at risk (Cusumano *et al.*, 1990).

Aspergillus fumigatus Most of the information on hand about *Aspergillus* infections has originated from the study of *A. fumigatus*, the most common species in the genus. Most of the Aspergillus infections are caused by *A. fumigatus* (Pasqualotto 2008). A variety of underlying conditions, including impaired immune status

contribute to the development of aspergillosis (Saral, 1991). *Aspegillus fumigatus* causes both invasive and allergic aspergillosis in patients with poor immune status (Hawksworth *et al.*, 1995). One of the most abundant metabolites produced by *A. fumigatus* during invasive hyphal growth is Gliotoxin. This toxin exerts a broad range of immunosuppressive effects in vitro, including inhibition of cytokine production, antigen presentation and production of reactive oxygen species by macrophages, and reduced cytotoxicity in T-cells (Kupfahl *et al.*, 2007).

Aspergillus niger is a filamentous ascomycete fungus that is present everywhere in the environment and is involved in opportunistic infections of humans (Perfect *et al.*, 2001). It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. (Samson *et al.*, 2001). Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins (Abarca *et al.*, 1994). When inhaled A*spergillus niger* can cause hypersensitivity reactions such as asthma and allergic alveolitis (Edwards and Al-Zubaidy, 1977).

Mucor Species in the genus *Mucor* have a cosmopolitan distribution and they can be found on almost any organic material that is in contact with air. *Mucor sp.* have been isolated from substrates such as soil, decomposing plant material, animal droppings, from air samples and in one case as a parasite on other fungi (Hesseltine, 1955). This specie may cause mucrosis in immunocompromised individuals. The site of infection are the lung, nasal and sinus passage (Crissy *et al.*, 1995).

Fusarium solani is common soil-borne fungi and a pathogen to many agriculture crops such as citrus (Nemec, 1987), beans (Silbernagel and Mills 1990) and peppers (Fletcher, 1994). *Fusarium* species are ubiquitous in soil (Booth, 1971). *Fusarium solani* is the most common Fusarium species found in humans and animals. It is an etiologic agent in keratitis, endophthalmitis, cutaneous infections in burn patients and mycetoma, finger nail infections, inflammation of sinus lining, pulmonary disease, endocarditis (inflammation of heart membrane), catheter infections, and septic arthritis (Anaissie *et al.*, 1997).

1.5.4. Brine shrimp assay:

Biologically active compounds are almost always toxic at high dose. Pharmacology is basically toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Thus in vitro lethality in a simple zoologic organism can be used as a suitable screen for selection and fractionation in the discovery and monitoring of biologically active compounds (McLaughlin *et al.,* 1998).

There is a tendency these days that demands substituting the use of laboratory animals in toxicological studies because of the high expenditure and the animals suffering caused by these tests. The other methods include procedures that could replace the experiments carried out with animals, reduce the number of animals used in every test, or improve the existing methodology in order to decrease pain and trauma (Zutphen and Balls 1997).

The brine shrimp lethality assay is thought to be a suitable method for initial evaluation of toxicity, finding fungal toxins, heavy metals and pesticides (Meyer *et al.,* 1982).

Brine shrimps can be used in a laboratory bioassay in order to determine toxicity by the estimation of the medium lethal concentration i.e. LC50 (Meyer *et al.,* 1982) which have been reported for a series of toxins and plant extracts (Lagadic and Caquet 1998).

The eggs of brine shrimp *Artemia salina* are easily available as fish food in pet shops. The eggs hatch within 48 hours in artificial sea water, producing large number of larvae. These tiny shrimp larvae (**Fig.1.6**) have been extensively used as a means to test the cytotoxicity of samples under study. This is a rapid, low-cost general bioassay which has been developed for screening, fractionation and monitoring of physiologically active natural products (Meyer *et al.,* 1982)

Brine shrimp (*Artemia salina*)

Fig.1.6. Brine shrimp (*artemia salina* larvae)

(www.eggcorns.lascribe.net)

1.5.5. Antitumor Assay:

The Crown Gall tumor (potato disc) assay can be used as a quite fast, low-cost and consistent pre-screen for antitumor activity (McLaughlin, 1991). The inhibition of crown gall tumors on the discs of potato tubers has an evident correlation with compounds and plant extracts known to be active in 3PS (*in vivo*, murine leukemia) antitumor assay. Thus by using the potato disc antitumor assay we can examine purified compounds for their tumor inhibition activity (Glasky *et al.*, 1980; Glasky *et al.*, 1981). The validity of this bioassay is predicted on the observation that certain tumor producing mechanisms are similar in plants and animals (Braun, 1972; Becker, 1975).

Agrobacterium tumefaciens is a ubiquitous soil borne pathogen responsible for Crown Gall disease, affecting many higher species of plant. It is an important problem for agriculture around the world. DNA transfer from *Agrobacterium tumefaciens* to eukaryotic cells is the only known example of inter-kingdom DNA transfer (Dumas *et al.*, 2001).

1.5.5.1. Crown Gall disease:

Crown Gall disease is a disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. These masses (tumors) may be spongy or hard, and may or may not have a deleterious effect on the plant. The tumors produced are histologically similar to those tumors found in humans and animals (Agrios, 1997).

When plant material is infected with *A. tumefaciens*, a tumor-producing Ti-plasmid, present in the bacterial DNA becomes integrated into the plant's chromosomal DNA. When plant tissue is injured it releases phenols, etc., which will activate the Ti plasmid in *A. tumefaciens*. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation. These tumors are similar to human and animal cancers in nucleic acid content and histology (Agrios 1997). After the integration of T-DNA to the plant genome which originates from the bacterial Ti (tumor inducing) plasmid there occur a set of genetic modifications in plants. Expression of these genes leads to the formation of Opines. These are specific oligosaccharides used solely by *A. tumefaciens* as a carbon source. After that expression of several oncogenic genes result in the formation of tumours (Gaudin *et al.*, 1994).

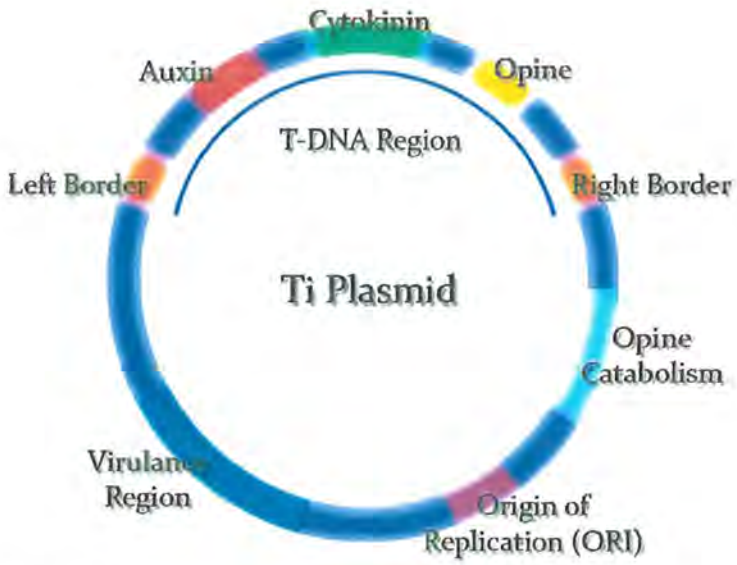


Fig.1.7. Ti Plasmid

Vir genes:

The processing and transfer of T-DNA are mediated by products encoded by the vir region where vir stands for virulance, which is also present on the Ti-plasmid (Stachel and Nester 1986). Those vir genes, whose products are directly involved in T-DNA processing and transfer, are strongly regulated so that expression occurs only in the presence of wounded plant cells i.e. the targets of infection. The vir region is of approx 35 kbp and is organized into six operons, namely vir A, vir B, vir C, vir D, vir E, and vir G. Control of gene expression is mediated by the VirA and VirG proteins (a two component regulatory system) (Tzfira and Citovsky, 2000).

Right and left border sequences:

The integration of the T-DNA into the plant genome depends upon specific sequences that are located at the right border of the T-DNA. The right border contains a repeating unit that consist of 25 base pairs. Although the left border contains a similar 25 bp repeat, it is evident from the deletion studies that this region is not involved in the integration process (Glick and Pasternak 2003).

Opines:

T-DNA also encodes enzymes for the synthesis of novel amino acid derivatives called opines. The Ti-plasmid encodes enzymes for their catabolism; hence, *Agrobucferium* has evolved to genetically commandeer plant cells and use them to produce compounds that they uniquely can utilize as a carbon/nitrogen source. Additional profit derives from the ability of opines to stimulate conjugation of the Ti-plasmid, thereby increasing the bacterial population that can utilize opines (Greene and Zambryski 1993).

1.5.6 Antioxidant assay:

Oxygen is a crucial element for life but under certain conditions it can have severely adverse effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of chemical compounds, known as reactive oxygen species or ROS (Bagchi and Puri 1998). Molecular oxygen can accept a total of four electrons, one at a time, and the corresponding number of protons to generate two molecules of water. When this process occurs within living cells , different oxygen radicals are formed as intermediate products, including superoxide ($O_2^{\cdot-}$)

peroxide (O_2^{-2}), which normally exists in cells as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\dot{O}H$). Superoxide, peroxide, and the hydroxyl radical are considered the primary ROS. As they are highly unstable and quickly react with more electrons and protons, most of these are converted to water before they can damage cells. It has been estimated that only about 2 to 3 percent of the O_2 consumed by the respiratory chain is converted to ROS (Chance *et al.*, 1979).

Because production of these reactive oxygen species occurs in nature, cells have evolved a variety of enzymatic and non enzymatic mechanisms to protect themselves against ROS (Yu, 1994).

Enzymes involved in the elimination of ROS include,

- superoxide dismutases (SODs),
- catalase,
- Glutathione peroxidase (Fridovich, 1997).

1.5.6.1 Free radicals:

A free radical is defined as any atom, molecule, or compound that is extremely unstable because of its atomic or molecular structure (i.e. the allocation of electrons within the molecule). In order to attain a steadier and stable state, free radicals can pick a hydrogen atom from another molecule, can bind to another molecule, or interact in a variety of ways with other free radicals (Wu and Cederbaum, 2003). The equilibrium between the production of free radicals and the antioxidant defences is very important for health of individuals (Bagchi and Puri 1998). Antioxidants are substances that counterbalance or neutralize free radicals and their actions (Sies, 1996).

1.5.6.2 Oxidative stress:

Oxidative stress can be defined as a destruction in the balance between oxidant and reductant species in living organisms. The living cells are exposed to oxidants originating from a large variety of sources exogenous as well as endogenous. Exogenous sources include air pollutants, natural harmful gases, ozone and high concentrations or high pressure of oxygen, effects of ionizing and non-ionizing

irradiation, chemicals and toxins, and pathogenic bacteria and viruses (Menzal, 1994 ; Podda *et al.*, 1998; Halliwell, 2000).

Oxidative damage is implicated in the etiology of cancer, cardiovascular disease, and other degenerative disorders. It is proposed that free radical-mediated lipid peroxidation is involved in several diseases including cancer, drug-associated toxicity, rheumatoid arthritis, postischemic reoxygenation injury, and in the degenerative processes associated with aging (Horton and Fairhurst, 1987; Esterbauer and Cheeseman, 1987).

Because of the large variety of maladies that have been related to reactive oxygen species, it is quite important to find new antioxidants that could inhibit or prevent the effects of ROS (Koracevic *et al.*, 2001; Perez, 2006).

1.5.6.3. Free radical induced oxidative DNA damage assay:

DNA is the main target of free radical damage. The types of damages induced are :

- Strand breaks, which can be single or double strand breaks
- Different forms of base damage yielding products such as 8-hydroxyguanosine, thymine glycol or abasic sites,
- Damage to deoxyribose sugar as well as DNA protein cross links.

These damages can bring about mutations that are heritable changes in the DNA that can cause cancer in somatic cells or malformations of foetus in the germ cells. The involvement of free radicals with tumour suppressor genes and proto-oncogenes imply their role in the development of different forms of human cancers (Halliwell and Aruoma, 1993). Among several molecules affected by a prooxidant status, DNA is of exclusive importance, because it is the storehouse of genetic information and is present in single copies. Different types of DNA mutations are produced by reactive oxygen species. The most common of these are base modifications. More than 20 of such modifications have been determined (Dizdaroglu, 1992).

The highly electron-dense structure of DNA makes it an extremely good target for attack by many free radicals, especially by the hydroxyl radical which is a very short-lived, reactive, and electrophilic species (Pryor, 1988).

The ability of the hydroxyl radical to damage DNA has therefore been the focus of extensive investigation in recent years (Daniel *et al.*, 1998). Lesions produced in DNA

after the hydroxyl radical attack have been detected by 32P-postlabeling (Randerath *et al.*, 1991; Carmichael *et al.*,1992). There are numerous mechanisms by which ROS might be generated in living organisms. One such mechanism is the Fenton reaction (Halliwell and Gutteridge, 1985), in which hydrogen peroxide is reduced by a transition-metal ion to form the hydroxyl radical (Fenton, 1894).

Fenton's reaction:

In 1894 H.J.H Fenton discovered that several metals have a special oxygen transfer properties which improve the use of hydrogen peroxide. Actually, some metals have a strong catalytic power to generate highly reactive hydroxyl radicals ($^{\cdot}OH$). Since this discovery, the iron catalyzed hydrogen peroxide has been called Fenton's reaction.

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + {}^{\cdot}OH + OH^-$$

http://www.lenntech.com/fentonsreaction.htm

Antioxidant and prooxidant effects can be determined using a free radical-induced plasmid pBR322 DNA breaks system in vitro. When -OH ion generated from the Fenton reaction attack super coiled plasmid DNA it is broken into three forms, including

- Super coiled (SC)
- Open circular (OC)
- Linear form (Linear).

The degree of DNA damage can be represented by the percentage of SC form in plasmid DNA bands, and the antioxidant or prooxidant effect of test samples can be explained by the ratio of SC percentage of test sample to that of the control i.e. DNA treated with $FeSO_4$ and H_2O_2 (Tian and Hua, 2004).

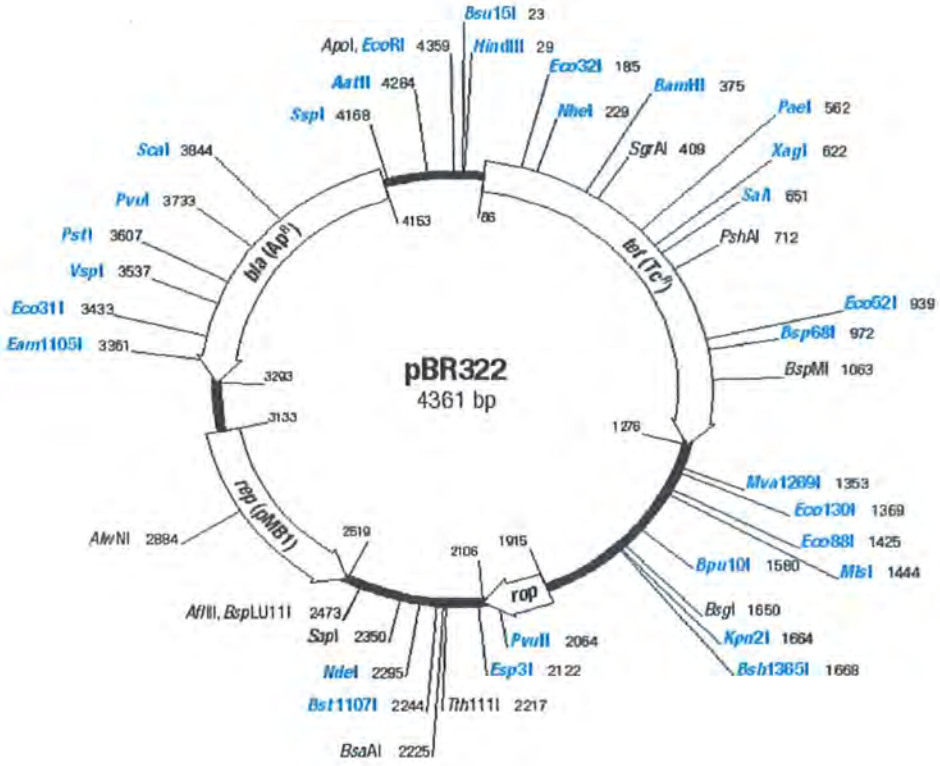
The plasmid pBR322 (**Fig.1.7**) is one of the most commonly used *E.coli* cloning vectors. pBR322 is 4361 bp in length. It contains:

(1) the replicon *rep* responsible for the replication of plasmid (source – plasmid pMB1)

(2) *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I – RNA II complex to a stable complex and serves to decrease copy number (source – plasmid pMB1)

(3) *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source – transposon Tn3)

(4) *tet* gene, encoding tetracycline resistance protein (source – plasmid pSC101).



The map shows enzymes that cut pBR322 DNA once.

Fig.1.8. Restriction map of pBR322 (Fermentas) plasmid

(http://dwb4.unl.edu/Chem/CHEM869N/CHEM869NLinks/www.fermentas.com/techinfo/NucleicAcids/mappbr322.htm)

Research Objectives

Objective of this research work was to evaluate a series of novel 1,4-disubstituted-1,2,3-triazoles for their possible antimicrobial (antibacterial and antifungal), antitumor, OH radical induced oxidative DNA damage/protection, and cytotoxic properties.

MATERIALS AND METHODS

This whole research work was conducted in the Molecular Biology Laboratory, Department of Biochemistry, Quaid-i-Azam University Islamabad. The compounds 1,4-disubstituted-1,2,3-triazole were synthesized by organic chemistry section of Quaid-i-Azam University Islamabad. A brief account of general procedure of synthesis and structures of synthetic compounds are described below.

2.1 Compounds used for the study:

Triazoles have been documented as significant antifungal and bacteriostatic compounds, so new 1,4-disubstituted-1,2,3-triazole were synthesized and screened for their biological activities. The present study aimed at the investigation of antimicrobial (antibacterial and antifungal), antitumor, OH radical induced oxidative DNA damage/protection and cytotoxic activity of the 15 novel compounds.

2.1.1 General procedure for synthesis:

1. 4-Acetylphenylazide (0.805 g, 5.0 mmol), and 1-ethynyl-1-cyclohexanol (0.62 g, 5.0 mmol)/ phenyl acetylene (0.55 mL, 5.0 mmol), were suspended in *tert*-BuOH/H_2O (4:1, 20 mL).
2. To this mixture was added $CuSO_4 \cdot 5H_2O$ (0.06 g, 0.25 mmol) and (+)-sodium ascorbate (0.3 g, 1.5 mmol).
3. The mixture was stirred at room temperature for 24 hours.
4. The reaction was monitored by TLC which indicated complete conversion.
5. The resulting solution was concentrated under reduced pressure.
6. The residue was dissolved in 40 mL of brine solution and then extracted with ethyl acetate (3 x 30 mL).
7. The combined organic layers were washed with 5 % aq. NH_4OH (2 x 25 mL), dried over $MgSO_4$, filtered and the solvent was removed under vacuum.
8. The crude product was recrystallized from ethanol to give pure product.

(Kolb *et al.*, 2001; Rostovtsey *et al.*, 2002; Efange *et al.*, 1991; Tornøe *et al.*, 2002; David, 2001)

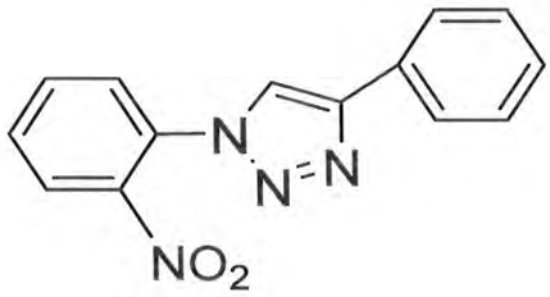
Table 2.1: List of 1,4-disubstituted-1,2,3-triazole

No.	IUPAC Names	Designated as
1	1-(2-Nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole	CC-1
2	1-(4-Nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole	CC-3
3	1-(2-Cyano-4-nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole	CC-4
4	1-(2-Acetylphenyl)-4-phenyl-1*H* 1,2,3-triazole	CC-5
5	1-(3-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole	CC-6
6	1-(4-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole	CC-7
7	1-(2-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-8
8	1-(3-Nitrophenyl)-4-(1-hyroxycyclohexyl)-1H-1,2,3-triazole	CC-9
9	1-(4-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-10
10	1-(2-Cyano-4-nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-11
11	1-(4-Acetylphenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-14
12	1-Hexadecyl-4-phenyl-1H-1,2,3-triazole	CC-15
13	1-Hexadecyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-16
14	1-Octyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-17
15	1-Benzyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole	CC-20

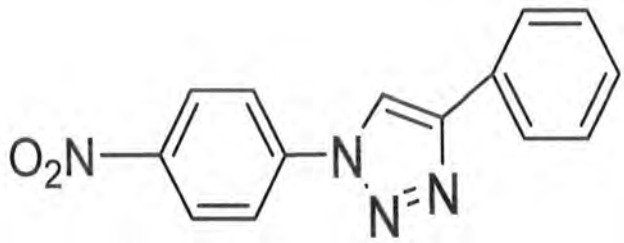
2.1.2 Structures of Compounds:

The structures of all 15 compounds are given below.

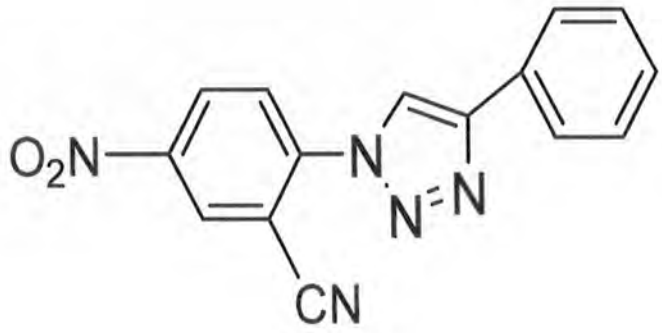
1-(2-Nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole (CC1)



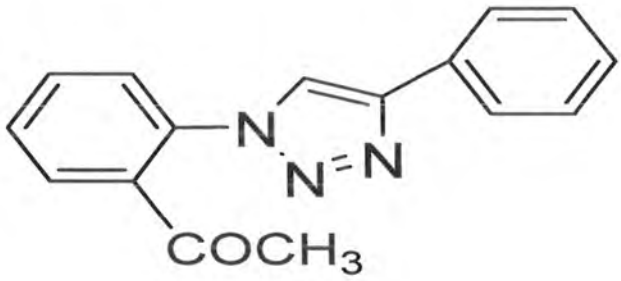
1-(4-Nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole (CC3)



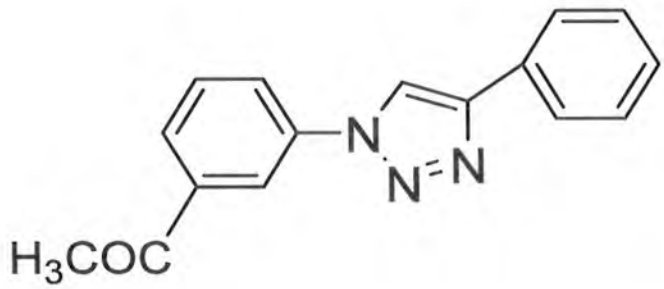
1-(2-Cyano-4-nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole (CC4)



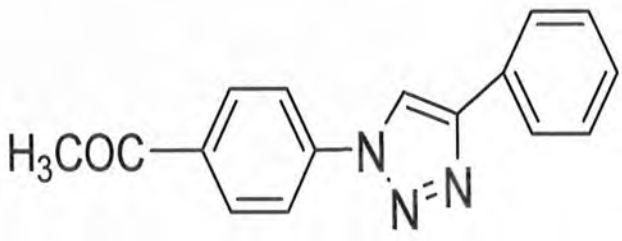
1-(2-Acetylphenyl)-4-phenyl-1*H* 1,2,3-triazole(CC5)



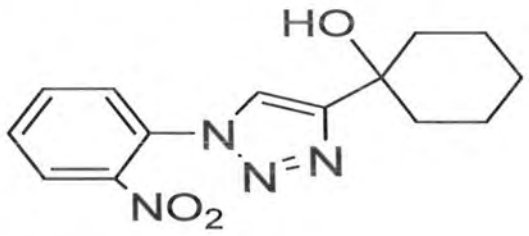
1-(3-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole (CC6)



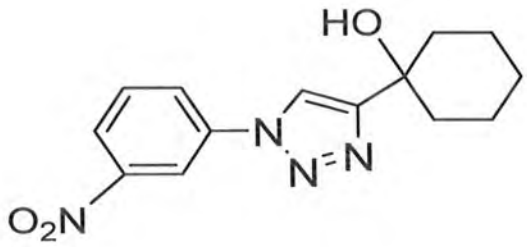
1-(4-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole (CC7)



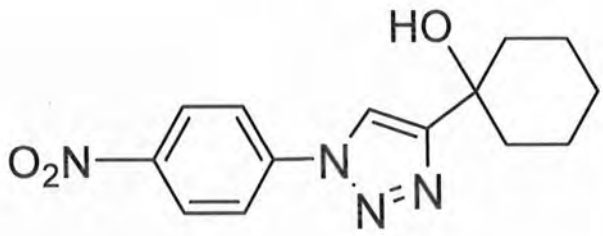
1-(2-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC8)



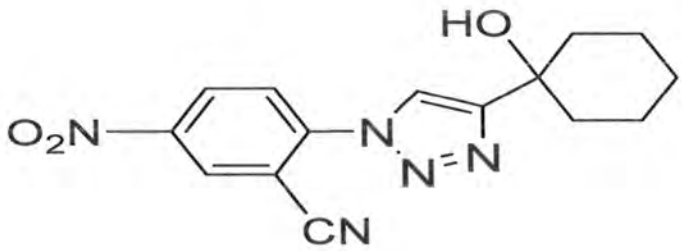
1-(3-Nitrophenyl)-4-(1-hyroxycyclohexyl)-1H-1,2,3-triazole (CC9)



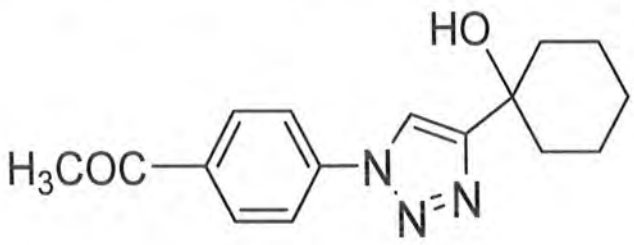
1-(4-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC10)



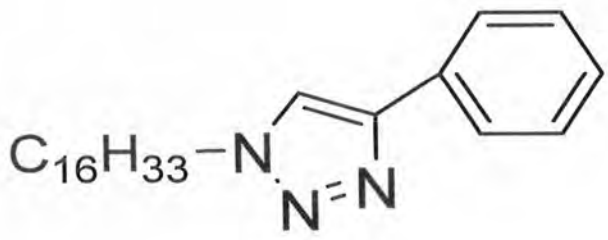
1-(2-Cyano-4-nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC11)



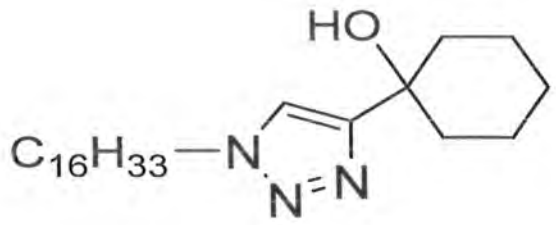
1-(4-Acetylphenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC14)



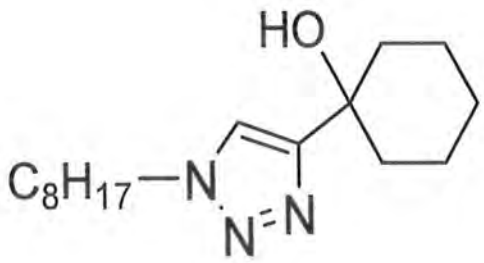
1-Hexadecyl-4-phenyl-1H-1,2,3-triazole (CC15)



1-Hexadecyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC16)



1-Octyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC17)



1-Benzyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole (CC20)

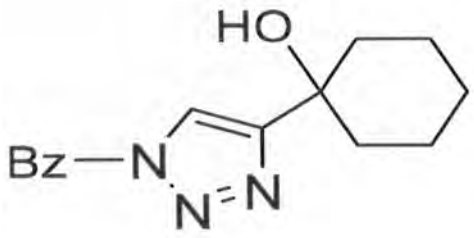


Fig. 2.1 structures of 15 newly synthesized 1,4-disubstituted-1,2,3-triazoles.

2.2 Sterilized Conditions for Handling Microbe Related Experiments

Conditions for handling microbe related assays like antimicrobial (antibacterial, antifungal) and antiumor assays were sterilized by taking following measures.

- Laminar flow hood and all the glassware used in the assay were swabbed with methylated spirit.
- Glassware is also autoclaved along with the growth media used in the assay.
- Whole experimental material was surface sterilized by giving UV treatment inside the Laminar flow hood.
- Spirit lamp was kept lighted during the experiment to kill bacterial and fungal spores around 10cm of lamp's diameter.
- Hands were also washed with spirit before the start of experiment and experiment was carried out in the laminar flow hood.

2.3 Antibacterial Assay:

All the test compounds were screened to determine their antibacterial activity by using ''agar well diffusion method'' (Olaleye, 2007).

2.3.1 Sample Preparation:

All compounds were dissolved in DMSO at a concentration of 1 mg/ml of DMSO.

2.3.2 Bacterial Growth Media:

Nutrient Broth (MERCK) was used as a media to grow bacteria for the inoculum preparation.

Nutrient Broth has following composition:

- Peptone (from meat) 5 gm/L
- Meat extract 3 gm/L

Nutrient broth medium was prepared by dissolving 0.8gm of nutrient broth in 100 ml of distilled water. Its pH was adjusted to 7.0 and was autoclaved.

Nutrient Agar (MERCK) was used as medium to grow bacteria in plates. It has following composition:

- Peptone (from meat) 5 gm/L
- Meat extract 3gm/L
- Agar-Agar 12gm/L

Nutrient agar medium was prepared by dissolving 2gm of nutrient agar in 100 ml of distilled water. Its pH was adjusted to 7.0 and was autoclaved.

2.3.3 Turbidity standard for bacteria:

In order to compare the turbidity of bacterial culture McFarland 0.5% $BaSO_4$ solution was used. 0.5 ml of 0.048M $BaCl_2$ (barium chloride) solution was added to 99.5 ml of 0.36N H_2SO_4 solution and this solution was used as the standard. Barium sulphate turbidity standard (4 to 6ml) was taken in screw capped test tube and was used to compare the turbidity of bacteria.

2.3.4 Bacterial Strains used:

All the synthesized compounds were tested for their antibacterial activity against five bacterial strains. Three were gram negative and two were gram positive strains.

Gram negative strains used were,

1. *Brodetella bronchiseptica* (ATCC 4617)
2. *Escherichia coli* (ATCC 15224)
3. *Enterobacter aerogenes* (ATCC 13048)

While gram positive strains were,

4. *Staphylococcus aureus* (ATCC 6538)
5. *Micrococcus luteus* (ATCC 10240)

Twenty-four hours old culture in nutrient broth (MERCK) of selected bacterial strain was mixed with physiological saline (0.9% NaCl w/v) and turbidity was corrected by adding sterile physiological saline until a McFarland 0.5 $BaSO_4$ turbidity standard [10^6 colony forming unit (CFU) per ml density] was obtained. Then this inoculum was used for seeding the nutrient agar.

2.3.5 Assay Procedure (agar well diffusion method):

Nutrient agar medium was prepared by suspending nutrient agar (MERCK) 2 gm in 100 ml distilled water. pH was adjusted at 7.0 and was autoclaved. Media was allowed to cool down to 45°C. Then it was seeded with 1 ml of prepared inoculum to have 10^6 CFU per ml. Petri plates (14 cm) were prepared by pouring 75 ml of seeded nutrient agar and were allowed to solidify. Eleven wells per plate were made with sterile cork borer (8mm). Using micropipette, 100µl of test solution was poured in its respective well. Eight samples two solutions for positive control (Roxithromycine 1 mg/ml and Cifixime-USP 1mg/ml, one for each) and one for negative control (DMSO) was applied to each Petri plate. In the end Petri plates were incubated at

37°C for 24 hours. After 24 hours diameter of clear zone (showing no bacterial growth) around each well was measured with the help of vernier calliper. Triplicate plates were prepared for each sample.

2.4 Antifungal Assay

Antifungal assay was performed on the test compounds by using 'Agar tube dilution method'(Choudhary *et al.*, 1995).

2.4.1 Fungal strains used:

Fungal strains used in this experiment are as follows:

1. *Mucor species* (ATCC 0300)
2. *Aspergillus niger* (ATCC 0198)
3. *Aspergillus flavus* (ATCC 0064)
4. *Aspergillus fumigatus* (ATCC 66)
5. *Fusarium solani* (ATCC 0291)

Each fungal strain was maintained on sabouraud dextrose agar medium at 4°C.

2.4.2 Media for Fungal Growth:

Sabouraud dextrose agar was used as medium to grow fungus for inoculum preparation. Its composition was:

- Peptone complex 10 gm/L
- Glucose 40 gm/L
- Agar 15 gm/L

2.4.3 Sample preparation:

Twelve mg of each compound was dissolved in 1 ml of DMSO to prepare initial stock solution. In this way the final concentration of 200µg/ml was obtained. DMSO was used as negative control. Twelve mg of turbinafine per ml of DMSO was used as positive control (standard drug).

2.4.4 Assay Procedure:

Media for fungus was prepared by dissolving 6.5 gm/100mL in distilled water. pH was adjusted at 5.6. Test tubes were marked up to 10 cm. The sabourad dextrose agar (MERCK) dispensed as 4ml volume into cotton plugged test tubes which were then autoclaved at 121°C . Test tubes were allowed to cool and in this non solidified SDA 67µl of test compound from the stock was added. This addition gave the final concentration of 200µg/ml of the pure compound in the media. Tubes were then allowed to solidify in slanting position at room temperature. The assay was carried out in triplicates i.e. three tubes for each compound were prepared. The tubes containing solidified media and test compounds were inoculated with 4mm piece of inoculum, taken from a seven days old culture of fungus. Positive and negative control test tubes with turbinafine and DMSO were also inoculated. The test tubes were incubated at 28°C for 7 days. Cultures were examined twice weekly during the incubation.

After 7 days, linear length of fungus growth was measured (in mm). Growth inhibition was calculated with reference to negative control.

Formula for percentage inhibition is as follows:

$$\text{Percentage inhibition of ungal growth} = 100 - \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in control}} \times 100$$

2.5 Brine Shrimp (Cytotoxicity) Assay

Brine shrimp lethality assay (McLaughlin *et al.,* 1998) was performed to check the cytotoxicity of test compounds.

2.5.1 Sample preparation

Sample was prepared by dissolving 2mg of test compound in 2 mL of methanol, DMSO and Chloroform according to the solubility requirement. This would make 1000ppm stock solution. From the stock solution further dilutions (100ppm and 10ppm) were made as given in the **fig.2.2**

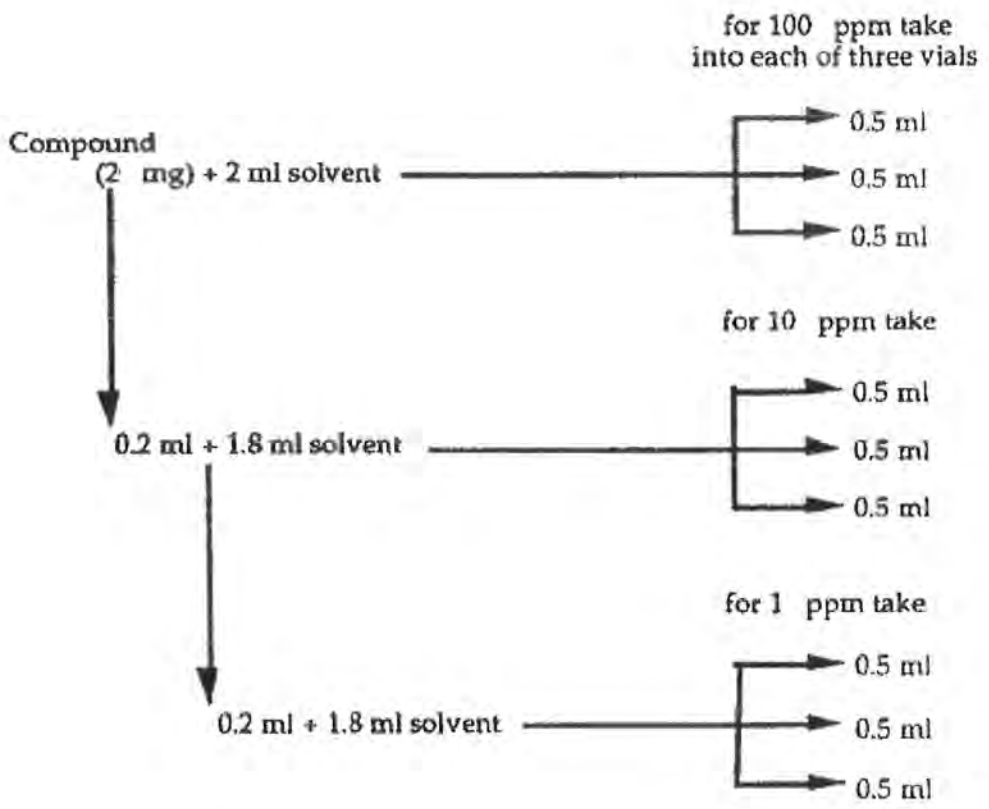


Fig.2.2 Preparation of dilutions for brine shrimp lethality assay.

2.5.2 Preparation of artificial seawater.

Artificial seawater was prepared by dissolving 34gm commercial sea salt in 1 liter distilled water with continuous stirring. It was aerated for two hours by vigorous shaking on magnetic stirrer.

2.5.3 Hatching shrimps

Brine shrimp (*Artemia salina*) eggs (Sera, Heidelberg, Germany) were hatched in shallow rectangular dish (22x32 cm) filled with prepared seawater. A plastic divider of 2mm with several holes was clamped in the dish to make two unequal compartments. The eggs (about 25mg) were sprinkled in the larger compartment, which was covered with aluminum foil to keep the eggs in dark, while the smaller compartment was illuminated. After 24 hours phototropic nauplii (brine shrimp larvae) moved towards light through the tiny holes in the divider which were collected by pipette from the lightened side.

2.5.4 Assay Procedure:

Two-dram vials were used for this bioassay. 0.5 ml of the each solution (1000ppm, 100ppm and 10ppm) was taken in the vial and methanol and chloroform was vacuum evaporated and residue was re-solubilized in 2ml of seawater. However for compounds dissolved in DMSO volume was raised to 5 ml before the DMSO toxicity would affect the results. Ten shrimps were transferred to each vial using Pasteur pipette, and raised the volume up to 5 ml. It made the concentration of each vial 100ppm, 10ppm and 1ppm respectively. Three replicates were prepared for each concentration.

The nauplii were counted macroscopically in the stem of Pasteur pipette against a light background. The vials were maintained under illumination at room temperature 25°C to 28°C. Survivors were counted with the aid of 3x magnifying glass after 24 hours.

2.5.5 ED_{50} Determination:

Percentage mortality was calculated. Then ED_{50} was calculated graphically by using finney computer programme (Probit analysis).

2.6 Antitumor (Potato Disc) Assay

The potato disc method was used for antitumor activity of test compounds as reported by (Ferrigini *et al.*, 1985).

Four different concentrations were prepared for each compound; these are 1000ppm 100ppm and 10ppm and 1ppm.

Microorganisms Used:

At10 strain of *Agrobacterium tumefaciens* was used.

2.6.1 Preparation of Bacterial Culture:

1. Luria broth medium was prepared by dissolving 2.5gm in 100ml of distilled water to make 2.5% solution. pH was adjusted at 7.0 and was autoclaved.
2. Twenty micro litres of 50mg/mL rifampicin stock solution was added to it. In the final volume concentration of rifampicin was 10µl/ml.

3. Single colony from culture plate of *Agrobacterium tumefaciens* (At10) was inoculated in it.
4. It was allowed to grow for 48 hours at 28°C in shaking incubator.

2.6.2 Preparation of Stock Solution of Compounds:

1. Stock solution was prepared by dissolving 1 mg of each compound in 1ml of DMSO (1000 ppm).
2. From the stock solution further dilutions of 100ppm 10ppm and 1ppm were made in DMSO.

2.6.3 Preparation of inoculum:

In order to prepare final concentration of 0.1ppm, 1ppm, 10ppm and 100ppm 1.5 ml of inoculum as prepared from initial stocks by adding 150µl of each solution was added in three autoclaved eppendorfs. Then 750 µl of autoclaved distilled water and 600µl o bacterial culture was added to it, so that final concentration of inoculum was 1500µl.

2.6.4 Preparation of Control Solutions:

Positive control:

Positive control was prepared by taking 150µl of DMSO in eppendorf and adding 1350µl of autoclaved distilled water in it.

Negative control:

Negative control was prepared by taking 150µl of DMSO in an autoclaved eppendrof and adding 750µl of autoclaved distilled water and 600µl of bacterial (At10) culture in it.

2.6.5 Preparation of Media:

Agar solution was prepared by dissolving 15gm/L agar in distilled water and was autoclaved. Three plates were prepared for each concentration of each compound and control. Agar solution was poured in autoclaved petriplates and allowed to solidify. 25mL of 1.5% agar was poured in each plate.

2.6.6 Preparation of Potato Discs:

1. Potatoes were soaked in 0.1% $HgCl_2$ (mercuric chloride) solution for 10 minutes.
2. After 10 minutes potatoes were taken out and rinsed with distilled water.
3. With the help of an 6mm borer cylinders were made out of potatoes.

4. These cylinders were washed again with distilled water in a large Petri plate and 1 cm piece from each end of cylinder was cut with sterilized blade and cut ends were discarded.
5. About 5mm thick disc were cut from these cylinders in another autoclaved Petri plate.
6. Discs were washed with distilled water and were placed on the solidified agar. Ten disc were placed per plate.
7. A volume of 50µl of inoculum form each concentration of each compound as well as from controls were added on the surface of each disc.

Petri plates were incubated in dark at 28°C for 21 days.

2.6.7 Staining The Potato Discs:

1. Lugol's solution was used for staining of the potato discs.
2. Lugol's solution consists of 10% KI and 5% I.
3. Stain was allowed to penetrate in the disc for few minutes.
4. Discs were observed under dissecting microscope with side illumination of light.
5. Destained portions of the discs were tumors. Number of tumors per disc was counted.
6. Percentage inhibition for each concentration was determined as follows:
7. Percentage inhibition = 100- $\frac{\text{average number of tumors of sample}}{\text{Average number of tumors of negative control}}$ X 100
8. 20% tumor inhibition was taken as significant inhibition.

2.7 Oxidative DNA damage analysis

Determination of antioxidant and prooxidant activity of synthesized organic compounds were conducted according to the method reported by Tian and Hua (Tian and Hua 2004) with slight modification.

2.7.1 Preparation of Stock Solution of Compounds:

Samples were prepared by dissolving 3mg of the test compound in 1ml of methanol. Concentration of this stock was 3000ppm. From this stock solution further dilutions (300ppm, 30ppm) were prepared.

2.7.2 Preparation of Phosphate buffer (PO_4 buffer 50mM):

PO_4 buffer was prepared by dissolving 0.18 gm of NaH_2 PO_4 and 0.55gm of Na_2H PO_4 in 100ml of distilled water.

2.7.3 Preparation of $FeSO_4$ (2mM):

$FeSO_4$ was prepared by dissolving 56mg of $FeSO_4$ in 100mL of distilled water to obtain 2mM solution.

2.7.4 Preparation of H_2O_2 (30%):

H_2O_2 was prepared by diluting 35% H_2O_2 to get 30% H_2O_2.

2.7.5 Assay Procedure:

Plasmid DNA pBR322, 0.5µg/µl (purchased from Fermentas, Germany) was diluted two folds (0.5µg/3µl) using 50 mM phosphate buffers (pH 7.4). The reaction was conducted in microeppendroff tubes in a total volume of 15µl. Three micro litres of diluted plasmid DNA was transferred to the microeppendroff tube followed by 5µl of stock solution of the test compound at three different concentrations, 3000ppm, 300ppm and 30ppm stock solutions generated 1000ppm 100ppm and 10ppm concentrations in the final reaction mixture (**Table 2.2**). 3µl of 2mM $FeSO_4$ and 4µl of 30% H_2O_2 was added successively.

2.7.6 Negative Control Preparation:

Three micro litres of diluted plasmid DNA and 12µl of phosphate buffer were added in microeppendorff tube and used as negative control for DNA damage and positive control to check the DNA protection activity (**Table 2.3**).

2.7.7 Positive Control Preparation:

Three micro litres diluted plasmid, 3µl of $FeSO_4$ 4µl of H_2O_2 and 5µl phosphate buffer was added in microeppendorff tube. It was used as positive control for DNA damage **(Table 2.3)**.

2.7.8 The effect of compound if any was checked by adding compound with pBR322 DNA in the absence of $FeSO_4$ and H_2O_2 **(Table 2.3)**.

Table 2.2: Final concentration (15µl) prepared for oxidative DNA damage analysis

Stock conc. Of compound(ppm)	Stock solution (µl)	Diluted plasmid pBR322(µl)	$FeSO_4$ (µl)	H_2O_2 (µl)	Final conc. Of the comp.(ppm)
3000	5	3	3	4	1000
300	5	3	3	4	100
30	5	3	3	4	10

Table 2.3: Control Reaction Mixture Preparation (15µl)

Control	PO_4 buffer (µl)	Plasmid pBR322 Diluted(µ)	$FeSO_4$ (µl)	H_2O_2 (µl)	Compound(µl)
Positive control for DNA damage (X)	**5**	**3**	**3**	**4**	-
Control for the damage effect of compound on DNA (P+C)	**7**	**3**	-	-	**5**
Negative control damage/Positive control protection(P)	**12**	**3**	-	-	-

The Reaction mixture was incubated in dark at 37°C or one hour. After incubation plasmid DNA was run on 1% agarose gel. Agarose (1gm) was taken in 250 mL flask and then dissolved in 100mL of 1X TBE buffer (Tris boric acid EDTA) and heated in

microwave oven to get a homogenous mixture. After slight cooling to bearable temperature, 20µl of staining agent ethidium bromide was added to agarose. After gentle shaking the agarose was poured in the gel tray, and allowed to solidify for 30 minutes.

After solidification of the gel 1X TBE (gel running buffer) was poured in the gel tank so that both electrodes were submerged in buffer. After incubation 3µl of bromophenol blue (loading dye) was added to each reaction mixture and then the samples were loaded on gel wells containing TBE buffer and ethidium bromide.

Each reaction mixture with controls was run horizontally in 1 X TBE buffer at 100 volts for 1 hour in an electrophoresis apparatus. The gels were photographed under UV light. For each lane, a molecular marker (1 kb ladder, L) along with all three controls was run along with the various antioxidant treatments.

RESULTS

Five assays were performed to evaluate the biological activities of the 15 triazoles. Compounds were evaluated for their antimicrobial (antibacterial and antifungal), antitumor, OH radical induced oxidative DNA damage/protection, and cytotoxic activity. Results for each of these assays are summarized below.

3.1 Antibacterial assay:

Antibacterial activity of 15 compounds was analysed using agar well diffusion method. Five strains of bacteria were used in the study including three gram negative and two gram positive strains. Gram negative strains used were, *Brodetella bronchiseptica* , *Escherichia coli*, and *Enterobacter aerogenes* while gram positive strains were *Staphylococcus aureus* and *Micrococcus luteus*. DMSO was used as a solvent for all the synthesized compounds which were dissolved at a concentration of 1 mg/ml of DMSO (i.e. 1000 ppm). DMSO is a good solvent for a variety of compounds and it has no inhibitory effect on bacterial growth. The standard drugs used against these bacterial strains were Roxithromycin (R) and Cefixime (C). Agar plates containing bacterial cultures were incubated at 37 0C for 24 hours for the growth of bacteria. Zone of inhibition for all the compounds were measured with the help of a vernier calliper (Fig 3.1).

MIC (minimum inhibitory concentration) was determined for the compound with significant activity. Results of the assay are presented in the table **3.1**. Most of these compounds have not given any significant activity against bacterial strains as shown in the table **3.1**. Compound CC 17 however gave activity against four (i.e. *Brodetella bronchiseptica* (MIC 0.8 mg/ml), *Enterobacter aerogenes* (MIC 0.2 mg/ml) *Staphylococcus aureus* (MIC 0.4 mg/ml) and *Micrococcus luteus* (MIC 0.4 mg/ml)) of the total five strains used. However no activity was observed by this compound against *Escherichia coli.*

Table 3.1: Antibacterial activity of 15 synthetic compounds

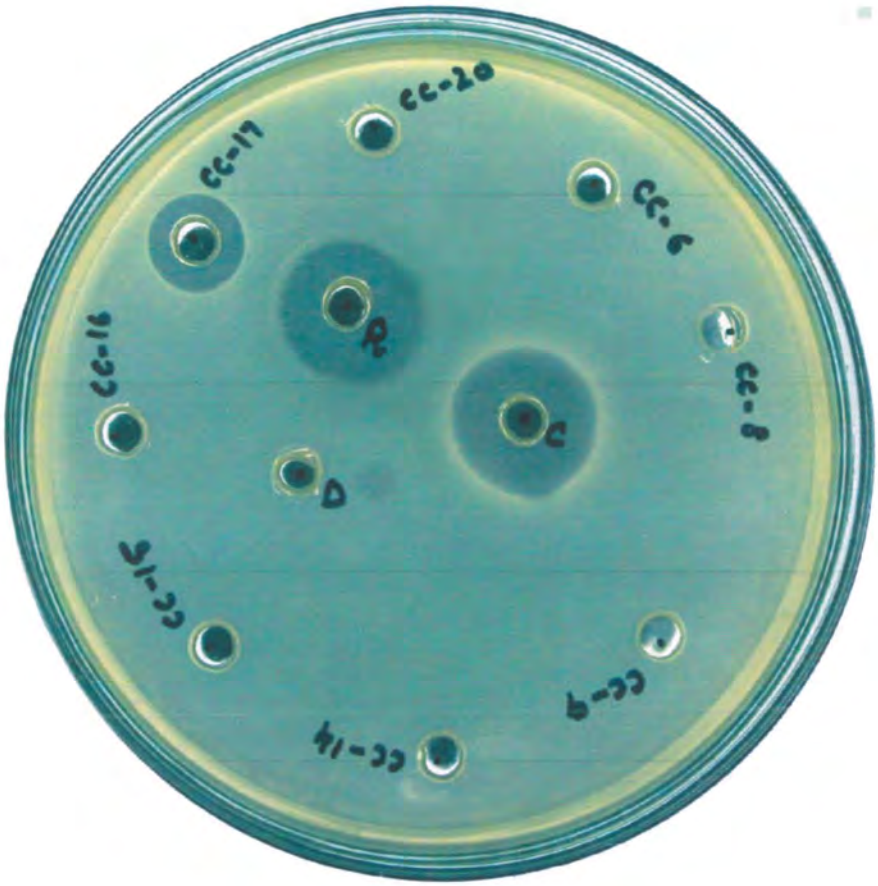
s.no	Sample name	Mean zone of Inhibition (in mm)+Mic				
		M. leuteus	***Entero-bactor aerogenes***	***E.coli***	***B.Bronchisep tica***	***S. aureus***
1	CC-1	Nil	Nil	Nil	Nil	Nil
2	CC-3	Nil	Nil	Nil	Nil	Nil
3	CC-4	Nil	Nil	Nil	Nil	Nil
4	CC-5	Nil	Nil	Nil	Nil	Nil
5	CC-6	Nil	Nil	Nil	Nil	Nil
6	CC-7	NIL	Nil	Nil	Nil	Nil
7	CC-8	Nil	Nil	Nil	Nil	Nil
8	CC-9	Nil	Nil	Nil	Nil	Nil
9	CC-10	Nil	Nil	Nil	Nil	Nil
10	CC-11	Nil	Nil	Nil	Nil	Nil
11	CC-14	Nil	Nil	Nil	Nil	Nil
12	CC-15	Nil	Nil	Nil	Nil	Nil
13	CC-16	Nil	Nil	Nil	Nil	Nil
14	CC-17	1.485mm(0.4 mg/ml)	1.155mm(0.2 mg/ml)	Nil	1.28mm(0.8mg/ ml)	1.27mm(0.4 mg/ml)
15	CC-20	Nil	NIL	Nil	Nil	Nil
	Roxithrom ycin	2.545mm	2.437mm	1.335mm	2.65mm	2.88mm
	Cefixime	3.55mm	3.14mm	3.23mm	2.29mm	3.33mm

M. Leuteus= Micrococcus leuteus, S. aureus = Staphylococcus aureus

B. bronchi =Bordetella bronchiseptica, E. coli= Escherichia coli.

Fig 3.1 Antibacterial activity of CC 6, CC 8, CC 9, CC 14, CC15, CC 16 and CC17 against

Micrococcus leuteus



R = Roxithromycin
C = Cefixime
D = DMSO

3.2 Antifungal assay:

Compounds were tested for their antifungal activity against five different fingal strains. Method used for the assay was agar tube dilution method. Linear inhibition in growth was observed for *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger Mucor sp. and Fusarium solani*. Turbinafine was used as standard drug that gave 100 % inhibition of fungal growth in all the strains used. Percentage inhibition of fungal growth was calculated with reference to negative control i.e. DMSO. Concentration of the compounds used was 12 mg/ml of DMSO. Compounds CC3, CC7, CC11 were not soluble in DMSO at this concentration so the assay was carried out on remaining 11 compounds.

Compounds CC17, CC5 and CC9 showed significant activity against different strains (**Fig.3.2**). Rest of the compounds gave varying degree of percentage inhibition (**Table3.3.2**). Level of significance was analysed by the criteria given in the **table 3.2.1**.

Table3.2.1: Growth inhibition and level of significant activity

Significance level	Growth inhibition
Low activity	Below 40% inhibition
Moderate activity	40-60% inhibition
Significant activity	60-70% inhibition
Good activity	70% and above

Formula used:

$$\%\text{age growth inhibition} = 100 - \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in negative control}} \times 100$$

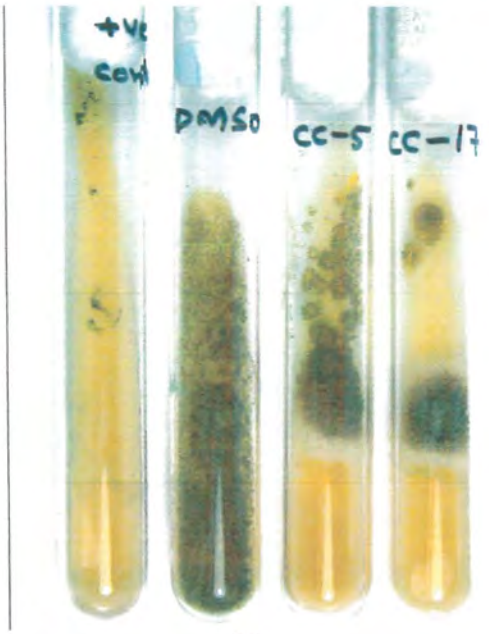
Table 3.2.2 Antifungal activity of 15 synthetic compounds.

s.no	Sample name	Percentage inhibition in linear growth				
		A. Niger %	*F.solani%*	*A.flavus %*	*A.fumigatus %*	*Mucor.sp. %*
1	CC-1	43	56	0.85	-	1.63
2	CC-4	26.31	40	11.11	-	-
3	CC-5	57.89	73.6	49.57	32	**55**
4	CC-6	3.1	53.6	6.83	-	4.91
5	CC-8	10.52	**56**	11.96	24	-
6	CC-9	30.52	**72**	13.6	28.7	17.21
7	CC-10	10.52	24	1.70	21.29	13.93
8	CC-14	13.68	48	9.4	13.8	46.72
9	CC-16	15.7	52	4.27	8.33	13.93
10	CC-17	61.05	80.8	100	63	34.42
11	CC-20	16.84	16	23.07	9.25	13.93

A.niger = *Aspergillus niger,* ***A.fumegatus*** =*Aspergillus fumegatus,*

A.flaves = *Aspergillus flavus,* ***F.solani*** = *Fusarium solani,* ***Mucor sp.*** = *Mucor specie.*

(A)



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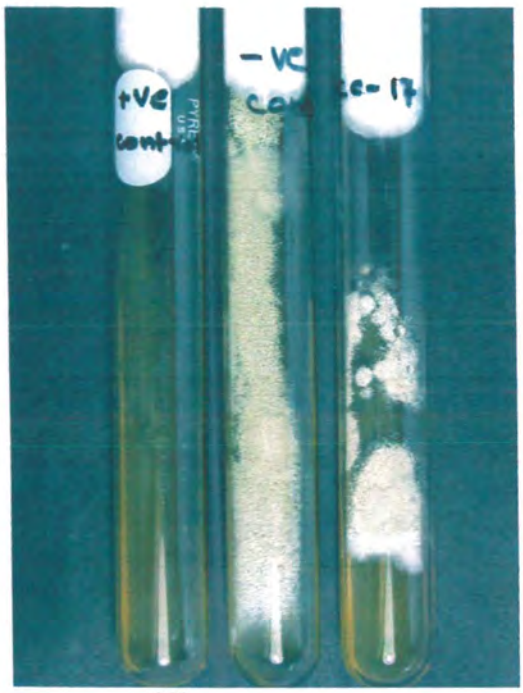

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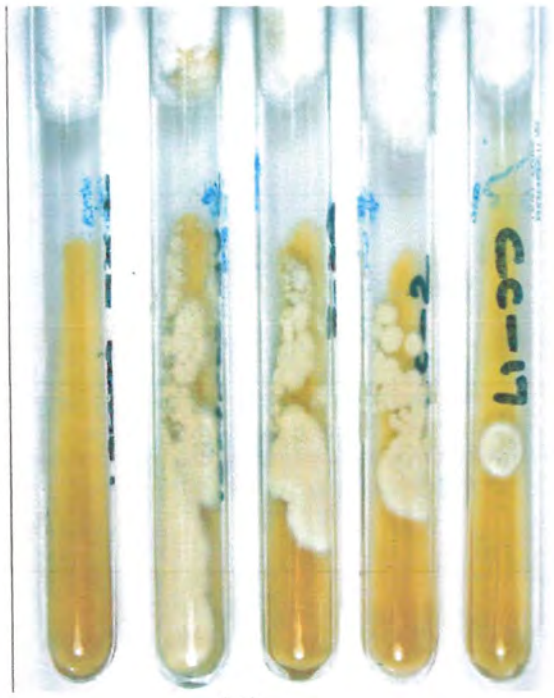

(D)

Fig 3.2: A study of antifungal activity.

A=Growth inhibition of *A. flavus* by CC5 and CC17
B=Growth inhibition of *A. niger* by CC5 and CC17
C=Growth inhibition of *A. fumigatus* by CC17
D=Growth inhibition of *F. solani* by CC9, CC5 and CC17.

3.3 Brine shrimp (Cytotoxicity) Assay

Brineshrimp assay was carried out on all 15 compounds to check them for cytotoxicity. Brine shrimp (*Artemia salina*) larvae were used for this purpose compounds were dissolved in DMSO, Methanol and Chloroform according to their solubility requirements. Three concentrations of each compound i.e. 1000ppm, 100ppm and 10ppm were prepared for the assay. After 1 day surviving shrimps were counted and data was analysed by Finney computer program (probit analysis) to determine the LD_{50} values.

Results of the assay are given in the **table 3.3.**

LD_{50} values indicate that compounds CC8, CC6 and CC16 has highly toxic effect on shrimp larvae because their LD_{50} values are 0.069ppm, 4.4ppm and 1.8ppm respectively. Compounds CC1, CC4 and CC14 have very high LD_{50} values which are greater than 1000ppm. Remaining compounds have moderate LD_{50} values.

LD50= lethality dose at which 50% shrimps are killed.

Concentration seemed to be an important factor for the activity of most of the compounds observed as in the case of CC11, CC16, CC17, CC20 all shrimps were killed at a concentration of 1000 ppm. The cytotoxic activity of these compounds decreased with decreasing concentration.

Table 3.3: Percentage mortality of brine shrimps at different concentrations of 15 compounds.

S.No.	Compounds	1000 ppm	100 ppm	10 ppm	LD50(ppm)
1	CC-1	24	12	8.04	>1000
2	CC-3	51	48.01	8.76	156
3	CC-4	65.54	39.9	36.01	>1000
4	CC-5	27.97	3.96	8.04	21.2
5	CC-6	8.04	39.6	3.99	4.4
6	CC-7	63.98	36.01	27.97	41.4
7	CC-8	15.9	15.9	27.97	.069
8	CC-9	81.08	63.98	24.00	62.2
9	CC-10	68.06	39.97	32.05	33
10	CC-11	100	65.37	50	9.1
11	CC-14	65.54	40.12	36.01	>1000
12	CC-15	96.18	92.30	50	5.56
13	CC-16	100	92.37	73.09	1.88
14	CC-17	100	65.35	53.81	7.17
15	CC-20	100	65.35	46.18	11.19

3.4 Antitumor assay:

Antitumor assay was performed on all the 15 compounds at four different concentrations. DMSO was used as a solvent for making 1 ppm, 10 ppm, 100 ppm and 1000 ppm concentration of each compound. While the final concentration became 0.1 ppm, 1 ppm, 10 ppm and 100ppm. DMSO and water served as positive control/Blank with no tumor growth whereas *Agrobacterium tumefaciens* (At 10) with DMSO dilution was used as negative control to calculate percentage inhibition. Tumor formation was observed after 21 days (**Fig.3.3**). Results are given in the table **3.4.1**.

Different compounds have shown dose independent activity. Some compounds have greater tumor inhibiting property at higher concentration these include CC5, CC9, CC 14. While other compounds are tumor inducing at higher concentration and have antitumor activity at lower concentration as seen in the case of CC1, CC8, and CC15. Compounds CC16 and CC14 are with highest percentage inhibition.

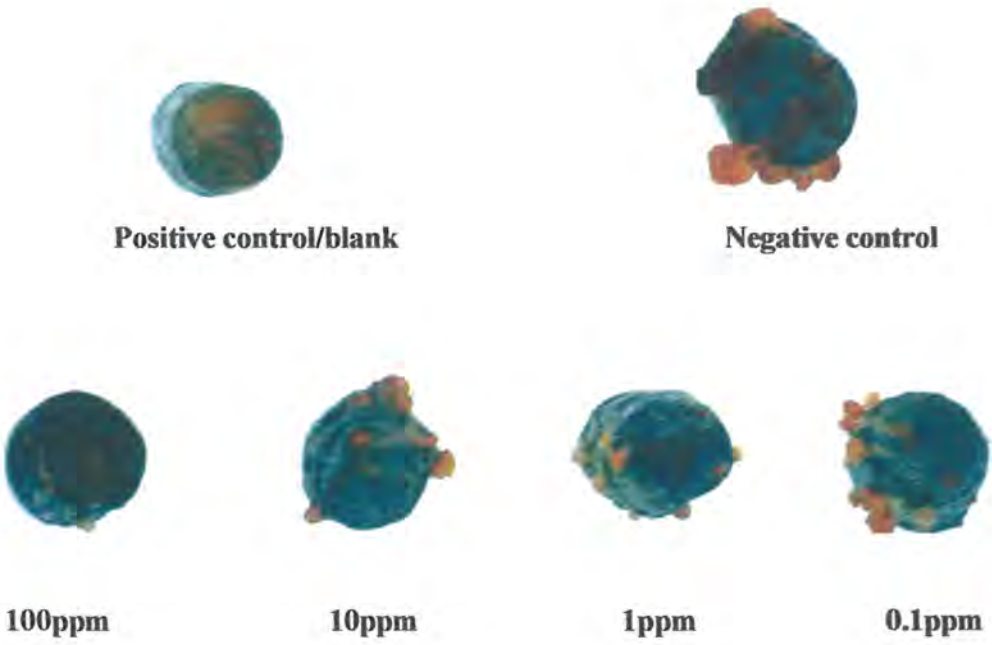

Fig 3.3: Tumor inhibition effect at three different concentrations of compound CC14.

Table 3.4.1: Percentage tumor inhibition by 15 compounds.

Compound		% Inhibition				IC50 ppm.
		100ppm	10ppm	1ppm	0.1ppm	
1	CC1	-38.18	14.54	32.72	58	-14.36
2	CC3	39.09	-14.54	-14.54	-8.18	121.76
3	CC4	20	28.81	16.36	12.72	1531
4	CC5	65.45	-22.72	10	-8.18	81.33
5	CC6	27.27	-18.18	-14.54	-75.45	131.04
6	CC7	-76.36	-38.18	-25.45	-17.27	-
7	CC8	-100	-63.63	51.81	60	-
8	CC9	57.27	-2.72	-10	-20	89.24
9	CC10	43.36	44	61.2	45.6	9.197
10	CC11	28	8.8	30	36	2586
11	CC14	81.2	51.2	60	46	-4.67
12	CC15	2	16.4	66	64	-3.21
13	CC16	62	77.6	77.8	81.6	167.2
14	CC17	51.6	-20.4	31.2	65.5	113.4
15	CC20	39.2	51.2	9.6	17.6	150.5

Percentage of inhibition: $100 - \frac{\text{No. of tumors with sample}}{\text{No. of tumor in negative control}} \times 100$

More than 20% tumor inhibition is significant.

Table 3.4.2: Analysis of variance (ANOVA) of tumor Inhibition of Compounds.

K value	Source	Degrees Of freedom	Sum of squares	Mean squares	F values	Probability
2	Compound (Factor A)	14	102495.482	7321.106	826.6283	0.0000
4	Concentration (Factor B)	3	6024.501	2008.167	226.7427	0.0000
6	AB	42	98862.824	2353.877	265.7769	0.0000
-7	Error	60	531.395	8.857		
	Total	119	207914.202			

Table 3.4.3: Effect of Compounds on percentage tumor inhibition.

Compounds	Mean values for inhibition*	Lsd Rank
CC1	16.85	G
CC3	0.3210	I
CC4	19.10	G
CC5	10.79	H
CC6	-20.23	K
CC7	-39.32	L
CC8	-12.96	J
CC9	6.138	HI
CC10	48.54	C
CC11	25.70	F
CC14	59.60	B
CC15	37.10	D
CC16	74.61	A
CC17	31.98	DE
CC20	29.53	EF

*Mean of % tumor inhibition at all concentration of same compounds.

Table 3.4.4: Effect of concentrations of compounds on percentage tumor inhibition and Lsd ranking of the concentration of the test compounds.

Concentration	Mean values for % inhibition**	Lsd rank
100ppm	20.14	A
10ppm	7.361	B
1ppm	25.34	A
0.1ppm	23.90	A

** Mean of percentage inhibition of all compounds at that concentration.

3.4.1 Statistical Analysis:

3.4.1.1 Effect of compound:

Significant effect of some compounds was observed on tumor formation. Most effective results were obtained in the case of compounds CC16 and CC14 (**Table 3.4.3**). Compounds CC15 and CC10 have moderate inhibition effect on tumor growth. Least inhibition was shown by compound CC7.

3.4.1.2 Effect of Concentration:

Effect of concentration on tumor inhibition is not as pronounced at $P<0.05$(**Table 3.4.4**) as that of individual compounds. Highest inhibition is shown at 1ppm.

3.5 Oxidative DNA Damage Analysis: DNA Protection/Damage Assay

The effect of compounds on free radical induced plasmid (pBR322) DNA damage analysis was tested at three final concentrations. Antioxidant and prooxidant ability was checked by incubating plasmid DNA with H_2O_2 and $FeSO_4$.

Positive control (X) = pBR322 along with $FeSO_4$ and H_2O_2

Negative control (P) = pBR322 in phosphate buffer.

Strand breaks in super coiled DNA result in change of conformation of DNA. A single stranded break converts a super coil in to an open circular form while double strand break converts it to the linear form. These three forms run differently on agarose gels.

The degree of DNA damage was observed from the gel patterns and intensity of each super coiled, open circular and linear bands formed after running the gel for 1 hour was observed. The compounds having antioxidant activity show more intense band of super coiled form of DNA.

Compounds CC1 and CC8 have shown good DNA protection activity at 3000ppm concentration. Compounds CC5 and CC9 have moderate DNA protection activity at all concentrations. Compounds CC6 and CC14 have shown good protection at 30 and 300 ppm respectively (**Table 3.5**). Remaining compounds have not shown any DNA protection activity, however, they don't have any DNA damaging effect, when incubated with plasmid only.

Table3.5: Comparison of DNA damage/DNA protection activity of ten compounds.

s.no.	compound	Concentration of compounds in reaction mixture		
		3000ppm	300ppm	30ppm
1	CC1	+++	+	++
2	CC5	++	++	+
3	CC8	+++	++	++
4	CC9	++	++	++
5	CC15	–	–	–
6	CC16	–	–	–
7	CC17	–	+	–
8	CC14	–	++	–
9	CC6	–	–	++
10	CC20	+	–	–

- \- DNA damage activity (no protection at all)
- \+ Low DNA protection activity
- ++ Moderate DNA protection activity
- +++ Good DNA protection activity

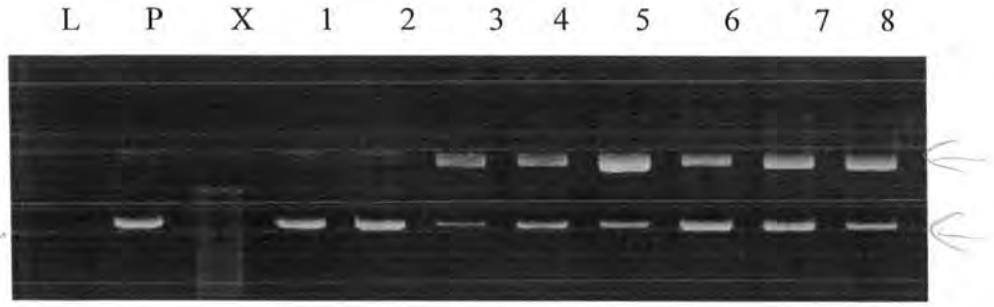

Fig 3.4: Effect of compounds CC1 and CC5 on pBR322 Plasmid DNA.

L: DNA Ladder (1 kb)

P: Plasmid (negative control damage/positive control protection)

X: +ive control of DNA damage

1: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC1

2: Plasmid+3000 ppm of CC1+$FeSO_4$+H_2O_2

3: Plasmid+300 ppm of CC1+$FeSO_4$+H_2O_2

4: Plasmid+30 ppm of CC1+$FeSO_4$+H_2O_2

5: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC5

6: Plasmid+3000 ppm of CC5+$FeSO_4$+H_2O_2

7: Plasmid+300 ppm of CC5+$FeSO_4$+H_2O_2

8: Plasmid+30 ppm of CC5+$FeSO_4$+H_2O_2

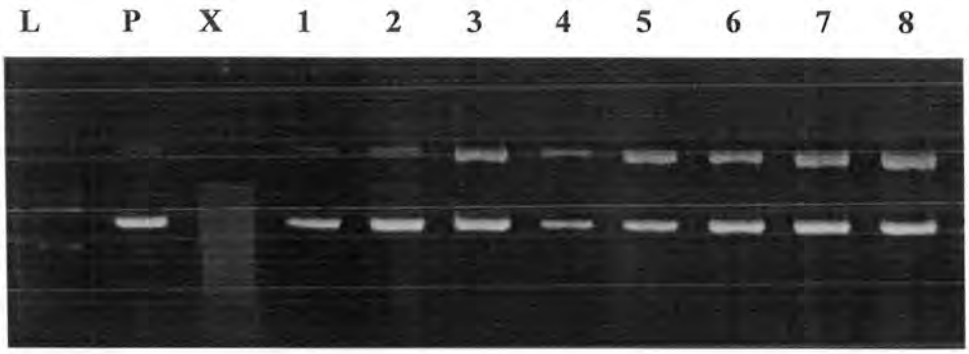

Fig 3.5: Effect of compounds CC8 and CC9 on pBR322 Plasmid DNA.

L: DNA Ladder (1 kb)

P: Plasmid (negative control damage/positive control protection)

X: +ive control of DNA damage

1: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC8

2: Plasmid+3000 ppm of CC8+$FeSO_4$+H_2O_2

3: Plasmid+300 ppm of CC8+$FeSO_4$+H_2O_2

4: Plasmid+30 ppm of CC8+$FeSO_4$+H_2O_2

5: Control for damage effect of compound on DNA ;plasmid+3000 ppm of CC9

6: Plasmid+3000 ppm of CC9+$FeSO_4$+H_2O_2

7: Plasmid+300 ppm of CC9+$FeSO_4$+H_2O_2

8: Plasmid+30 ppm of CC9+$FeSO_4$+H_2O_2

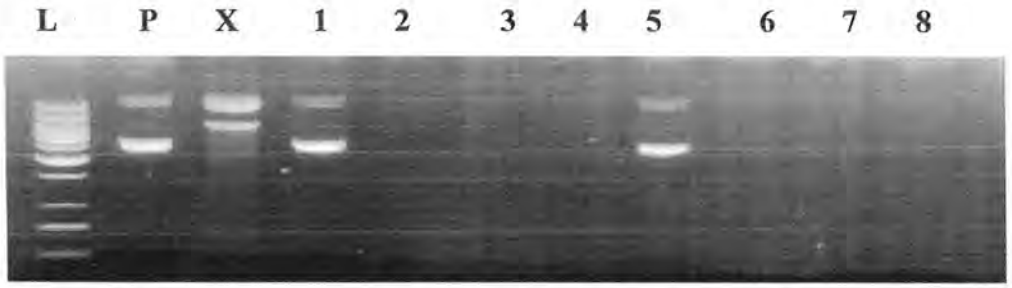

Fig 3.6: Effect of compounds CC15 and CC16 on pBR322 plasmid DNA.

L: DNA Ladder (1 kb)

P: Plasmid (negative control damage/positive control protection)

X: +ive control of DNA damage

1: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC15

2: Plasmid+3000 ppm of CC15+$FeSO_4$+H_2O_2

3: Plasmid+300 ppm of CC15+$FeSO_4$+H_2O_2

4: Plasmid+30 ppm of CC15+$FeSO_4$+H_2O_2

5: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC16

6: Plasmid+3000 ppm of CC16+$FeSO_4$+H_2O_2

7: Plasmid+300 ppm of CC16+$FeSO_4$+H_2O_2

8: Plasmid+30 ppm of CC16+$FeSO_4$+H_2O_2

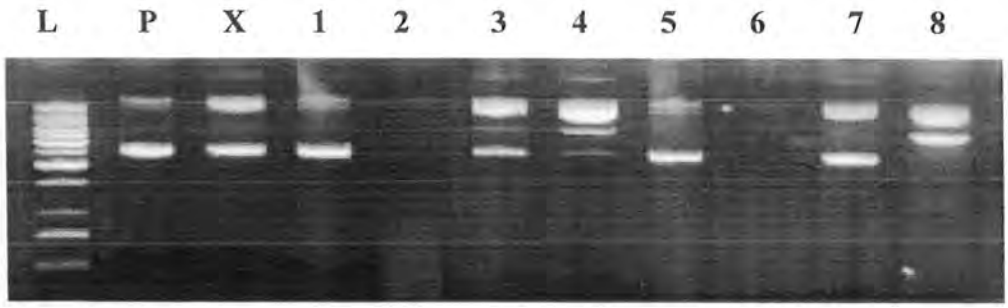

Fig 3.7: Effect of compounds CC17 and CC14 on pBR322 Plasmid DNA

L: DNA Ladder (1 kb)

P: Plasmid (negative control damage/positive control protection)

X: +ive control of DNA damage

1: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC17

2: Plasmid+3000 ppm of CC17+$FeSO_4$+H_2O_2

3: Plasmid+300 ppm of CC17+$FeSO_4$+H_2O_2

4: Plasmid+30 ppm of CC17+$FeSO_4$+H_2O_2

5: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC14

6: Plasmid+3000 ppm of CC14+$FeSO_4$+H_2O_2

7: Plasmid+300 ppm of CC14+$FeSO_4$+H_2O_2

8: Plasmid+30 ppm of CC14+$FeSO_4$+H_2O_2

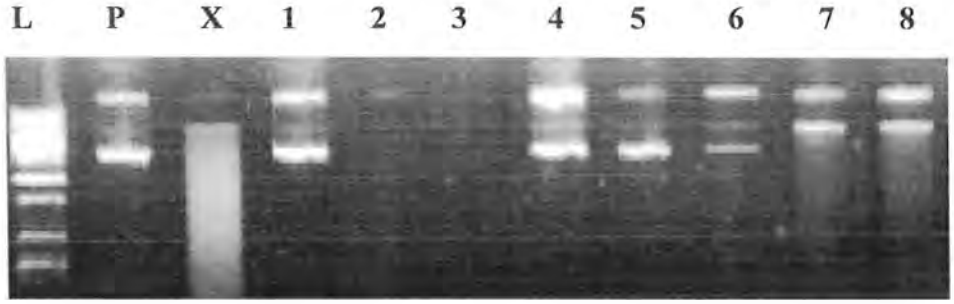

Fig 3.8: Effect of compounds CC6 and CC20 on pBR322 Plasmid DNA

L: DNA Ladder (1 kb)

P: Plasmid (negative control damage/positive control protection)

X: +ive control of DNA damage

1: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC6

2: Plasmid+3000 ppm of CC6+$FeSO_4$+H_2O_2

3: Plasmid+300 ppm of CC6+$FeSO_4$+H_2O_2

4: Plasmid+30 ppm of CC6+$FeSO_4$+H_2O_2

5: Control for damage effect of compound on DNA; plasmid+3000 ppm of CC20

6: Plasmid+3000 ppm of CC20+$FeSO_4$+H_2O_2

7: Plasmid+300 ppm of CC20+$FeSO_4$+H_2O_2

8: Plasmid+30 ppm of CC20+$FeSO_4$+H_2O_2

Discussion

In living organisms effect of a drug results from the interaction of certain drug molecules with macromolecules within the body like proteins and nucleic acids. This knowledge revolutionized the pharmacological industry and a new search for the pure chemicals instead of crude extracts began. However discovery of new drugs is still a tedious job because of lengthy drug development procedures and the high risk of unexpected side-effects. The process of drug discovery involves the identification of candidates, synthesis, characterization, screening, and assays for therapeutic efficacy. Once a compound has shown its value in these tests, it will begin the process of drug development prior to clinical trials.

In the search of new therapeutic compounds fifteen novel 1,4-disubstituted-1,2,3-triazoles were synthesized and screened for their possible biological activities including antimicrobial, antitumor, antioxidant and cytotoxic activities.

4.1 Antibacterial assay:

All newly synthesize compounds were screened for their antibacterial activity by agar well diffusion method. Except for the compound CC17, none of these compounds has shown any antibacterial activity against gram positive and gram negative bacteria. This behaviour of compounds is a little different from the findings of Reck *et al.*, (2005), where the authors report that 1,2,3-triazoles having a substituent like methyl, or small substituted methyl group at position 4 are good antibacterial compounds. The moderate activity of compound CC17 can be attributed to the presence of hydroxycyclohexyl group present at position 4 along with octyl group at position 1. This arrangement is not present in any other compound of the series except for CC16(1-Hexadecyl-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole) but the difference between the two compounds is that the alkyl group in case of CC16 is more bulky (hexadecyl) than that of CC17.

Compound CC17 (1-Octyl-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole) has shown activity against four *(Brodetella bronchiseptica, Enterobacter aerogenes Staphylococcus aureus and Micrococcus luteus)* of the five strains tested. But the activity is not as marked as to be used pharmacologicaly. More over no activity was found against *E. coli* bacteria this result is according to the previous findings (Al Bay *et al.*, 2010).

4.2 Antifungal Assay:

Fungal infections in past few years have become one of the major causes of disease mostly because of increase in the number of immune compromised patients, long term use of steroids, immunosuppressive drugs, and a haphazard use of antibiotics (Fridkin and Jarvis 1996; Larocco and Burgert 1997; Patel and Paya 1997).

1,2,3 triazole can be considered as a new entry in already present azole antifungals. The molecular mechanism of triazole antifungal activity is similar to the action of imidazoles.In the metabolic fungal pathways there are unique sites of action for the azole cvompounds.The primary mechanism being the inhibition of ergosterol biosynthesis which result in the accumulation of 14-alpha-methylsterols, the precursor intermediates of ergosterol (Bossche *et al.,* 1980; Bossche *et al.,* 1983; Bossche *et al.,* 1985; Brogers and Van de Ven 1987).

The compounds were screened for their possible antifungal activity against five fungal strains (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger Mucor sp. and Fusarium solani*).

Significant antifungal activity was shown by the compounds CC1, CC5, CC6, CC8, CC9, and CC17 against different fungal strains. Highest antifungal activity was shown by the compound CC17 **(1-Octyl-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole)** against *A. flavus* (100%). Same compound has shown significant activity against *F.solani* (80%), and *A. fumigatus* (63%). This activity of the compound can be because of presence of hydoxy cyclohexyl group at position 4 along with an alky group at position 1. These results are in accordance with the findings of Singh (2009) who claimed that alky or substituted alkyl group is responsible for enhancing the antifungal activity.

The other important compound with antifungal activity was CC5 **(1-(2-Acetylphenyl)-4-phenyl-1*H* 1,2,3-triazole)** with significant activity against *A. niger, F. solani*, and *mucor sp.* This is similar to the previous findings that substituted aromatic compounds are more active in inhibiting fungal growth than unsubstituted aromatic compounds (Saini *et al.,* 2010).Although similar groups are attached to the triazole ring in case of compound CC6

(1-(3-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole) but the change in position of acetyl group on phenyl ring can be the reason for the loss in activity.

4.5 Brine shrimp (Cytotoxicity) Assay:

Biologically active compounds usually have toxic effect on Brine shrimp larvae. Therefore in vivo lethality to shrimp larvae can be used as a speedy and straightforward initial monitor for bioactive compounds (Meyer *et al.*, 1982).

In the present study all 15 synthetic compounds were tested for their cytotoxic activity using brine shrimp (*Artemia salina*) lethality assay. Compounds CC8 (**1-(2-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**), CC6 (**1-(3-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole**) and CC16 (**1-Hexadecyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**) have shown highly significant cytotoxic activity with their LD 50 values ranging from 0.06 to 4.4.

Compounds CC1, CC4 and C14 have least toxicity with their LD 50 values greater than 1000ppm.

Some compounds have shown greater toxicity at higher concentration these are CC11 (**1-(2-Cyano-4-nitrophenyl)-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**), CC16 (**1-Hexadecyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**), CC17 (**1-Octyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**), CC20 (**1-Benzyl-4-(1-hydroxycyclohexyl)-1H-1,2,3-triazole**) all these compounds have shown 100% mortality in shrimps larvae at a concentration of 1000 ppm.

All above stated compounds have hydroxycyclohexyl group in their structures and it can be the reason for their toxicity because compounds with highest LD50 values i.e. CC1, and CC4 do not posses hydroxycyclohexyl group in their structure.

Compounds CC6 and CC14 are exception in a way that former doesn't posses hydroxycyclohexyl group and have shown very good activity whereas compound CC14 do have the hydroxycyclohexyl group but is only slightly toxic.This varying cytotoxic activity among compounds is similar to previous findings of cytotoxic activity in triazoles (Bagihalli *et al.*, 2008).

4.3 Antitumor Assay:

The inhibition of *A. tumefaciens*-induced tumors (or Crown Gall) in potato disc tissue is an assay based on antimitotic activity and can detect a wide range of novel antitumor agents (McLaughlin and Rogers, 1998). Thus all 15 compounds were checked for their antitumor activity using potato disc tumor assay.

This series of 1,4-disubstituted-1,2,3-triazole has not shown very promising antitumor activity. A couple of compounds gave significant activity, these are compound CC14 **(1-(4-Acetylphenyl)-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole)** and CC16 **(1-Hexadecyl-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole).** Compounds CC15 **(1-Hexadecyl-4-phenyl-1*H*-1,2,3-triazole)** and CC10 **(1-(4-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole)** exhibited moderate activity against tumor formation. A look at structures of CC14, CC16, and CC10 shows that 1-hydroxycyclohexyl group is attached to the position 4 in all of these compounds so the difference in their tumor inhibiting properties could be because of the presence of different groups attached to position 1 of the triazole ring and position of substituent on these groups. These results confirm the previous findings of anticancer properties of 1,2,3 triazoles (Tian *et al.,* 2005) considering that certain tumorigenic mechanisms are similar in plants and animals (Becker, 1975; Braun, 1972; Karpas, 1982).

Some compound including CC7 **(1-(4-Acetylphenyl)-4-phenyl-1*H*-1,2,3-triazole)** have shown tumor enhancing effect instead of inhibition.

A comparison of cytotoxic activity and antitumor activity of compounds shows that compound CC14 is least toxic of all compounds but has very good antitumor activity. On the other hand compound CC 16 is highly toxic towards shrimp larvae and possesses very good antitumor acitvity as well.

4.4 Free radical induced DNA damage assay:

Antioxidants are very important for the health of an individual. In our bodies there is a balance between the production of free radicals and antioxidant defence mechanisms. If free radicals increase in number and there are too few antioxidants are present, oxidative stress can develope within the body. This oxidative stress is implicated in the various

health problems such as cancer, atherosclerosis, cerebrovascular accidents, myocardial infarction etc. (Bagechi and Puri 1998).

Free radicals and other reactive oxygen species can cause oxidative damage to DNA and can result in many complications within the body (Ames, 1983).

Antioxidants and prooxidant effects of synthesized compounds were investigated using free radical induced plasmid (pBR322) break system *in vitro*. The assay was carries out at three different concentrations of test compounds. OH^- radicals were produced using Fenton's reaction. Super coiled DNA is converted to three forms because of the attack of free radicals ,these three forms are supercoiled (SC), open circular (OC), and linear form (Linear). Effects of compounds were compared to the control (cleavage of DNA in the presence of H_2O_2 and $FeSO_4$), and thickness of super coiled and open circular bands was examined. Only Four of the total 10 compounds tested for antioxidant activity have shown DNA protacting effect. These compounds are CC1 **(1-(2-Nitrophenyl)-4-phenyl-1*H*-1,2,3-triazole)**, CC5 **(1-(2-Acetylphenyl)-4-phenyl-1*H* 1,2,3-triazole),** CC8 **(1-(2-Nitrophenyl)-4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazole)** and CC9 **(1-(3-Nitrophenyl)-4-(1-hyroxycyclohexyl)-1*H*-1,2,3-triazole).** These results are in accordance with the previously reported antioxidant potential of triazloes (Sayed *et al.*, 2010).

The protective effect was greater at higher concentration of the compound in the case of CC8 and CC1 but concentration had little effect on results in case of CC5 and CC9. Presence of Nitrophenyl group attached to the position 1 of three of these compounds indicate that this can be the reason for their protective effect against free radical damage to DNA. All other compounds did not show any noticeable DNA protective ability.

Conclusion:

Biological evaluation of 15 novel 1,4-disubstituted-1,2,3 triazoles was carried out to determine their antimicrobial, antitumor, antioxidant and cytotoxic properties. All compounds were lacking antibacterial activity against all five fungal strains used however compound CC17 exhibited some antibacterial activity which was absent against *E.coli*. Antifungal activity was shown by many compounds of the series. Highest activity was shown by compound CC17 against *A. Flavus*. Same compound exhibited good activity against other strains as well. Compounds CC5 and CC9 also

posses significant antifungal activity against some of the strains. Variable results were shown by the compound against tumor production in potato disc antituomor assay. Where compounds CC14 and CC16 gave the highest percentage inhibition. Some other compounds also gave percentage inhibition over 50 at different concentrations these include CC1, CC5,CC8 and CC15. DNA protection against free radical induced DNA damage was shown by compounds CC1, CC5, CC8 and CC9. Cytotoxic studies of these compounds reveals highly toxic effects by compounds CC6, CC8 and CC16.

In the light of above mentioned activities of present set of triazoles it can be concluded that some of the 1,4- disubstituted-1,2,3 triazoles can be potent antfungal agents. However they need to be further optimized to perform better as antitumor and antioxidant agents.These compounds can be screened further for other pharmacological activities to be used as potential drug candidates and their mechanism of action and structure activity relationships (SAR) can also be studied.

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