DOI: 10.21276/haya.2016.1.2.5

Haya: The Saudi Journal of Life Sciences **ISSN 2415-623X (Print)**
Scholars Middle East Publishers **ISSN 2415-6221 (Online)**
Dubai, United Arab Emirates
Website: http://scholarsmepub.com/

Research Article

Studies in the Antimicrobial Activities of Some Newly Synthesized [1,4] Substituted Benzodiazepines by Disc Diffusion Method and MIC Methods

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Abstract: Recently in this laboratory 8-phenylthiacabamido-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4] benzodiazepine i.e.**M-1**, 8-(phenyl-2,4-dithiabiureto)-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4] benzodiazepine i.e.**M-2**, 8-[(3-phenylimino)-1,2,4-dithiazolo]-amino-1-methyl-6-phenyl-4H-[1,2,4]triazolo [4,3-a][1,4] benzodiazepine i.e.**M-3**, 8-[(2-phenylimino-6-phenylamino)-1,3,5-dithiazinoimino]-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4]benzodiazepine i.e.**M-4**, 8-[(2-thio-3-phenyl-6-phenylamino)-1,3,5-thiadi- azino]imino-1-methyl-6-phenyl-4H-[1,2,4] triazolo [4,3-a] [1,4] benzodiazepine i.e.**M-5** were synthesized and their antimicrobial activities were tested against *E. coli, B. subtilis, S. typhi, S. aureus, B. megatherium, A. aerogenes, P. vulgaris* by Disc diffusion method and MIC methods. These compounds showed good results.
Keywords: Benzodiazepines, antimicrobial activities, *E. coli, B. subtilis.*

INTRODUCTION

Drug is defined as, "any chemical substance inhibiting the growth or causing the death of a micro-organism". Chemical agents not only provide the information about structural basis and energy supply to living organism but also regulate their functional activities. The interactions between potent chemicals and living system contribute to understand the life processes and provide effective methods for the treatment, prevention and diagnosis of many diseases. A chemical substances used for this purpose are referred as 'drugs' and their actions on living system are known as 'drug effect' which can be preliminary tested by counting the antimicrobial activities of any molecule. 1,4-Benzodiazepine nucleus is useful nucleus in the case of liposome in a potent carrier for targeted doxorubicin having high affinity for sigma receptor and used for the treatment of human malignancies including human prostate cancer cells. 1,4-Benzodiazepinedione has been reported as potent antagonist's interaction in vitro and in cell-based assays and also proved that they possess anti-consultant, anxiolytic, anti-tumor properties [1]. It is effective against cholecystokinin receptor (CCK), opiate receptor and platelet glycoprotein antagonists [2, 3]. Many derivatives of benzodiazepines are widely used as sedative, anti-depressive, anti-inflammatory and hypnotic agents [4-5]. It is also used as dyes for acrylic fibers [6]. Recently new series of 1,2,4-thiadiazoles, 1,3,5-thiadiazines and 1,3,5-dithiazines were synthesized by exploring the synthetic applications of -thiocarbamido, -amino, -halo, -cyano, etc. and their antimicrobial, antifungal, antibacterial, analgesic physiochemical parameters [7-10] were studied. Hence it was thought interesting to carry out the antimicrobial activities against various microbes to check the drug properties of newly synthesized 1,4-substituedbenzodiazepines by Disc diffusion method and MIC methods in this laboratory.

EXPERIMENTAL

The antibacterial activities of **M-1** to **M-5** compounds were tested to evaluate their efficiencies against pathogenic organisms. All the chemical and media were purchased from M/s. Hi-Media Pvt. Ltd., Mumbai, India. The organisms used were taken for studies *E. coli, B. subtilis, S. typhi, S. aureus, B. megatherium, A. aerogenes, P. vulgaris*.

For the evaluation of in-vitro antimicrobial activity, the following three conditions must be fulfilled,

i) First the substance to be evaluated must be brought in an intimate contact with the test organisms against which activity is to be estimated.
ii) Secondly, favorable conditions (nutritional, environmental etc.) must be provided to offer a

maximum opportunity for optimum growth of the organisms in absence of antimicrobial agent, and

iii) Thirdly, there should be a method for measuring antibacterial response obtained by antimicrobial agent.

Various methods had been proposed and adopted for the measurement of antibacterial activity, these are,

1. Agar streak dilution method.
2. Agar diffusion (cup, paper, disc, cylinder) method.
3. Turbidometric method.
4. Serial dilution method.
5. Specific method (specific for measuring the action of specific substance).

In the present study, we used agar disc diffusion method to find out the activity of all synthesized compounds against the microbes. Then the minimum inhibitory concentrations were measured by serial dilution method for those compounds only which were found to be active.

A) Media Used

1. Nutrtient Agar Medium - Composition

Yeast extract	1.5 gms
Beef extract	1.5 gms
Peptone	5.0 gms
NaCl	5.0 gms
Agar powder	20 gms
Distilled water	1000ml
pH	7.4±0.2(at25°C)

2. Nutrient Broth Medium – Composition

Yeast extract	1.5 gms
Beef extract	1.5 gms
Peptone	5.0 gms
NaCl	5.0 gms
Distilled water	1000 ml
pH	7.4±0.2(at25°C)

Both the above cited media used were of bacteriostatic grade. Above media were found to be suitable for the growth of all four organisms used in the present work.

B) Slant preparation

Nutrient agar medium was dissolved in distilled water and then sterilized by auto calving. About 5 ml of molten media was transferred aseptically in previously sterilized test tubes. The test tubes were then plugged tightly and placed in a slanting position to cool and solidify.

C) Stock culture

Culture was grown on nutrient agar slants by incubating them for 24 hours at 37°C.

D) Culture dilution (Sub-culturing)

One loopful of stock culture was added to 5 ml of nutrient broth medium for inoculation. The inoculated broth was incubated for 24 hours at 37°C. For all experimental purposes 24 hours fresh diluted culture of both the organisms were used.

E) Preparation of sample solution

An antibacterial activity is usually tested by making aqueous solution samples. However, compounds used in the present study are insoluble in water. Hence, to study antimicrobial activity their dilutions were prepared by using ethanol. Thus, ethanol was taken and tested as control.

To check the potency of compounds, the solutions were prepared with 50 alpha gm/ml concentration. 1 ml of this solution was added to 5 ml of nutrient broth solution containing organism to be tested. Tubes with organism and medium with solvent were used as controls. These tubes were kept for incubation at 37°C for 24 hrs. Most of the compounds under study exhibited total inhibition of the test cultures within 24 hours of incubation. The tube containing compounds showing inhibition (antimicrobial activity) was clear and the tube which was kept as control where no compound was added showed growth. Therefore, for all the antibacterial screenings, the concentrations of 50 gm/ ml was used, which is in the range of the substance to be used as antibiotic.

F) Disc diffusion method

Every time fresh sterile nutrient agar medium was prepared. The proceedings were carried out aseptically. All the glassware and apparatus required were sterilized. In each sterile Petridis 15-20 ml of molten medium was added. Simultaneously 0.05-0.1 ml (approx. 2-3 drops) of 24 hours fresh diluted culture of organism under study was added to each petriplate. The nutrient broth culture and nutrient agar media were mixed thoroughly by rotatory motion of agar plate on a plane surface. It was allowed to solidify at room temperature. Then sterilized Whatmann filter paper No. 1 discs (6 mm diameter) thoroughly moistened with the same concentration of each of the compound were placed on the surface of the plate. Disc moistened with ethanol was used as control. They were allowed to diffuse in the media and then the plates were incubated at 37°C for 24 hrs. The diameter of the zones of inhibition was observed.

The same procedure was followed for determining antifungal activity; only the potato dextrose plate was used.

3. Potato dextrose agar - Composition

Potato infusion form	200 gms
Dextrose	20 gms
Agar	15 gms
Distilled water	1000 ml
pH	5.6±0.2(at25°C)

The compounds, which showed antimicrobial activity, were further tested for their minimum inhibitory concentration by Serial Dilution Method.

G) Serial dilution method

To determine the MIC of various compounds the following procedure (Serial Dilution Method) was followed.

Nutrient broth was prepared by dissolving 13 gms of dehydrated medium in 1 liter of distilled water. The pH of the medium was adjusted to 7.4. 5ml of the medium was distributed in each tube. All the tubes were sterilized at 121°C for 20 minutes.

The appropriate amount of test compound was dissolved in the solvent ethanol gave final concentration of 1 x 10^{-2} M. Various amounts of the above stock solution was aseptically added to the various nutrient broth tubes (viz. 0.5, 1.0,1.2, 1.4, 1.6, 1.8, .0 ..... 5.8, 6.0 ml). Fresh culture of the test bacterium was inoculated in each tube (0.2 ml culture). The inoculums size of the test bacterium was adjusted to give approx. 107 cFu. All the tubes were incubated at 37°C for 24 hours. Uninoculated tube was kept as a control in which nutrient broth and 5 ml of the solvent was taken.

After 24 hours of incubation, all the tubes were observed for MIC against test bacterium. This was observed by the absence of visual turbidity in the tube receiving the highest dilution of the test compounds. To determine MIC of various test compounds against moulds (fungus) the following procedure was adopted. Potato dextrose broth was prepared as follows. 200 gm. of potato (Peeled) was added to 1 liter of distilled water. It was steamed for 20 min and volume adjusted to 1 liter. 20 gm of dextrose was added to this.

Appropriate amount of test compounds were dissolved in ethanol mixture to give final concentration of 1xl0-2 M. Various amounts of the above stock solution was added aseptically to the potato dextrose broth tubes (viz. 0.5, 1.0, 1.2,.... 6 ml). Fresh fungal culture was inoculated aseptically in each tube (0.2 ml of culture). All the tubes were incubated at 28°C for 96 hrs. After 48 hrs. of incubation all the tubes were observed for the MIC of test compounds.

RESULTS AND DISCUSSION

Total 05 synthesized compounds were studied for their antimicrobial activities. All the pathogens tested during analysis are human pathogens. The activities of compounds were tested against all the pathogens by disc diffusion method. It was found that all the compounds are active against *E. coli*, *B. subtilis*, *S. typhi*, *S. aureus*, while inactive against *B. megatherium*, *A. aerogenes*, *P. vulgaris* as shown in Table No-1. MIC values were measured for the active compounds only, and given in Table No.-2

Table 1: Activity of 1,4-substitutedbenzodizopines

Comp.	*E. coli*	*B. subtilis*	*S. typhi*	*S. aureus*	*B. megatherium*	*A. aerogenes*	*P. vulgaris*
M1	Active	Active	Active	Active	Inactive	Inactive	Inactive
M2	Active	Active	Active	Active	Inactive	Inactive	Inactive
M3	Active	Active	Active	Active	Inactive	Inactive	Inactive
M4	Active	Active	Active	Active	Inactive	Inactive	Inactive
M5	Active	Active	Active	Active	Inactive	Inactive	Inactive

Table 2: MIC values of active compounds in mgml^{-1}

Comp.	*E. coli*	*B. subtilis*	*S. typhi*	*S. aureus*
M-1	790	846	823	1161
M-2	670	449	363	723
M-3	460	1145	1785	1624
M-4	550	479	463	538
M-5	852	771	1043	1343

MIC values, Inactive-3500-1900, Weakly active-1800-1500, Moderately active-1400-1000, Highly active < 1000

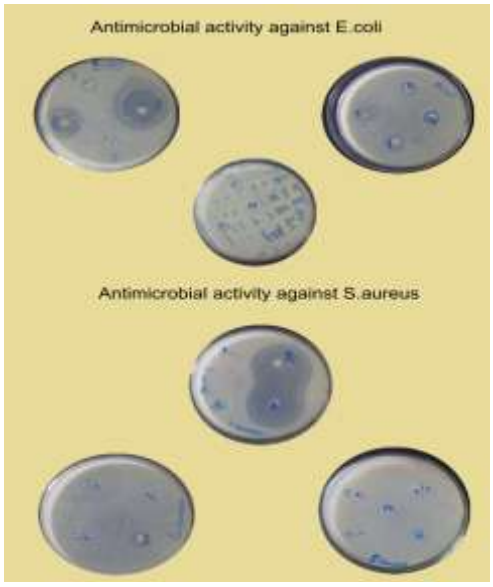

Fig-1: Antimicrobial activity

Activity against E. coli

E. coli is a gram negative parasite living only in human or animal intestine. The clinical infections caused by *E. coli* were urinary tract infection, diarrhea, pathogenic infection and septicemia. Generally the patients of diarrhea were observed in February to July. The treatment of these patients who suffers diarrhea, normally medicinal practitioners make use of sulphonamides, cotrimoxazole, quinolones, ampicillin, cloxacillin, ciperacillin, carbenicillin, cephalosporin, gentamycine, chloramphcnicol, tetracycline etc. antibiotics. These drugs directly effect on digestive system and ultimately circulatory system and finally on kidneys. Also the above drugs once used for the treatment of *E. coli* infection should not be used up to six months.

The antimicrobial activity of the synthesized compounds against E. coli is highly remarkable, all compounds are highly active.

Activity against *E. coli*

From, Table No.-2 it can be easily seen that the compounds M-3 and M-4 showed highly activity in minimum concentration. Compounds M-3 and M-4 contain 1,2,4-dithiazolo 1,3,5-dithiazino nucleus along with 1,4-benzodiazipine nucleus respectively these nuclei may be responsible for the higher activity of these compounds. Thiocarbamido, 2,4-dithiabiureto and 1,3,5-thiadiazino nucleus also increases activity of M-1, M-2 and M-5 respectively. So these synthesized drugs can be used as the best alternative drugs for the treatment of diseases caused by *E. coli*, only after the pharmaceutical, biochemical and medicinal significance, if these drugs do not have adverse and other toxic effects.

8-phenylthiacabamido-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4] benzodiazepine i.e.**M-1**, 8-(phenyl-2,4-dithiabiureto)-1-methyl-6-phenyl-4H-[1,2,4] triazolo [4,3-a] [1,4] benzodiazepine i.e. **M-2**, 8-[(3-phenylimino)-1,2,4-dithiazolo]-amino-1-methyl-6-phenyl-4H-[1,2,4]triazolo [4,3-a][1,4] benzodiazepine i.e. **M-3**, 8-[(2-phenylimino-6-phenylamino)-1,3,5-dithiazinoimino]-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4] benzo diazepine i.e. **M-4**, 8-[(2-thio-3-phenyl-6-phenylamino)-1,3,5-thiadi-azino]imino-1-methyl-6-phenyl-4H-[1,2,4] triazolo [4,3-a] [1,4] benzodiazepine i.e. **M-5**

All compounds are highly active against ***B. subtilis*** except **M-3** which is moderately active. M-1, M-2 and M-4 compounds are highly active, **M-5** is moderately active while, M-3 is weakly active against ***S. typhi***.

M-2 and M-4 compounds are highly active, M-1 and **M-5** is moderately active while M-3 is weakly active against ***S. aureus***.

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