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Study of carcinogenicity, Toxicity, and alterations of metal binding domains to susceptible value of experimental bacterial strains of some currently used antibiotics.

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ABSTRACT

The antibacterial effect of metal-based drugs has been reported in a number of publications. Copper phenanthroline complex has antibacterial activity against P. aeruginosa (Geraghty et al., 1999). Ames test for detection of carcinogenicity of 1,10-phenanthroline-5,6-dione and metal complexes using Salmonella typhimurium to determine the level of mutagenicity associated with metal binding domains (MBDs) was used in this study. Effect of MBDs on some strains of Gram-positive and Gram-negative bacteria was examined. The study determined the MIC90 of MBDs on bacteria and compared the effect with some currently used antimicrobial drugs. The effect of sub MIC90 concentrations of MBDs on the tolerance of bacteria to antibiotics was examined.

The aim of this work was to define the antibacterial activity of 1,10-phenanthroline, metal salt and metal complexes against a range of Gram positive and Gram negative bacteria. MIC90 value of metal-based drugs was determined against 7 type of bacteria and the viability of Gram negative and Gram positive bacteria was studied to determine the effect of MBDs on bacterial viability. Six types of currently used antibiotics were tested against each bacterium to determine the MIC90 and whether the metal based drugs altered the susceptibility of bacteria to antibiotic was investigated using microdilution toxicity assay.

Key words: metal-based drugs MBDs Ames test, MIC test, 1,10-phenanthroline

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Introduction

The aim of this work was to study the mutagenic properties of metal-based drugs. Use of metal-based drugs as antifungals has been reported in a number of publications. They have fungicidal and fungistatic properties (1). Metal binding domains (MBDs) have also antibacterial properties. 1, 10-phenanthroline and its derivatives were shown to be bactericidal nearly for five decades ago. The in vitro antibacterial action of 1,10-phen and metal complexes have been demonstrated on several species of bacteria, where they can be bacteriostatic (2) and bactericidal (3). The cytotoxicity of phenanthroline is thought to depend upon the chelation of transition metal (4, 5). In this work the mutagenic properties of MBDs were assessed since the use of MBDs therapeutically would depend on being non-mutagenic and with low toxicity. The environment is full of potential carcinogens (cancer-causing agents) such as UV light, industrial pollutants, pesticides, food additives and tobacco products. These carcinogens can induce cancer because they are mutagens (chemicals that cause mutation), which change the nucleic acid sequence of DNA. It is important to have available a rapid and inexpensive assay for testing the chemicals suspected as carcinogenic, as well as the large number of new synthetic chemicals being produced each year.

Chemicals that induce mutations increase the rate of change from the auxotrophic to the prototrophic form and this is reflected in an increase in the number of colonies that grow in the medium. It is estimated that 90% of all carcinogens are also mutagens and, with this in mind, Ames and his colleagues developed a test in the 1970s that uses bacteria that are very sensitive to mutagenic agents.

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The Ames test is now used to screen many chemicals rapidly and inexpensively. Those few chemicals that are mutagenic are tested further in animals to assess their ability to cause cancer. Wild-type cultures of the bacterium salmonella typhimurium grow in medium without the addition of any amino acids. This is possible because they have metabolic pathways for making all of their own amino acids. Each amino acid has a separate pathway for its synthesis. The tests are designed whether the compound can interact with DNA and lead to the production of gene mutations or chromosomal breakage. The detection of mutagens is important because they may also be either teratogens or carcinogens. The Salmonella typhimurium microsomal test developed by **Ames** (5) is the commonly used genotoxicity test.

Bacterial strain used:

Gram positive strains
Staphylococcus aureus (Microbiology Laboratory St. James Hospital, Dublin), Methicillin Resistant Staph Aureus (Microbiology Laboratory St. James Hospital, Dublin), Staphylococcus simulanes (Microbiology Laboratory Mater Hospital, Dublin and Micrococcus luteus (Microbiology Laboratory Mater Hospital, Dublin 7)

Gram Negative strains
Escherichia coli (Microbiology Laboratory Mater Hospital, Dublin 7), Bacillus oleronius (Microbiology Laboratory Mater Hospital, Dublin 7) and Pantonea agglumerans (Microbiology Laboratory Mater Hospital, Dublin 7)

Culture
Cultures of experimental strains grown on an agar plate until colony was forming, it is best to seal edges of the agar plate so that it does not dry out over time, store 2 to 8 0C . To maintain bacterial cultures, they must be aseptically transferred to new plate every 4 to 6 weeks to maintain viability. Tester strains for working experiment were cultured in Oxoid nutrient broth media over night at 30 0C, 200 rpm.

Chemicals
Chemicals were purchased from commercial sources and, unless specified, were used without further purification. Phendio was prepared in accordance with the literature method (Yamada, 1992). [Cu(phendio)3](ClO4)2·4H2O, to a pale blue solution containing Cu(ClO4)2·6H2O (0.37 g, 1.0 mmol) in ethanol (20 cm3) was added phendio (0.63 g, 3.0 mmol) and the resulting green solution was stirred at room temperature for 0.5 h. The precipitated green solid was filtered off, washed with ethanol and ether and then air-dried. Yield: 0.74 g (77 %). % Found: C, 44.93; H, 2.01; N, 8.74. % Calc: C, 44.85; H, 2.72; N, 8.71. IR (KBr): 3445, 1707, 1640, 1584, 1486, 1436, 1313, 1264, 1104, 932,729, 630 cm-1. Solubility: H2O, EtOH. [Ag(phendio)2]ClO4, to a solution of phendio (0.2 g, 1.0 mmol) in ethanol (20 cm3) was added AgClO4 (0.1 g, 0.4 mmol) and the resulting beige coloured solution was stirred at 30oC for 0.5 h. After cooling to room temperature the precipitated orange product was filtered off, washed with small volumes of ethanol and ether, and then air dried (with light excluded). The solid was recrystallised from hot acetonitrile. Yield: 0.43 g (69%)
% Found: C, 45.99; H, 1.91; N, 8.98. % Calc: C, 45.96; H, 1.93; N, 8.93. IR (KBr): 3432, 1695, 1640, 1578, 1467, 1430, 1307, 1104, 929, 818, 735, 627 cm-1. NMR: 1H NMR (ppm DMSO): 8.85 (dd, 2H), 8.46 (dd, 2H), 7.72 (dd, 2H). Solubility: hot MeCN, DMSO

Ames Test For Metal Based Drugs used in this Experiment:
Top agar [0.6 % (w/v) agar and 0.5 % (w/v) NaCl] was sterilised by autoclaving at 1200C at 15 pka for 20 minutes and kept in incubator at 45 0C. Top agar was supplemented with 10 % of 0.5 mM histidine/biotin solution immediately prior to use. Two ml of top agar was distributed into a sterile universal tube and held at 450C. The mammalian enzyme activation system consisted of the S9 liver fraction of a male Sprague-Dawley rat, Rat liver S9 (Acroclor-1254-induced) was used for this experiment to prepare the standard S9 mix. In fume hood 500 µl of S9 mix (4 %, v/v), 100 µl of test compound at the appropriate concentration and 100 µl of tester strains 0.6 absorbance at 605 nm were added to two ml aliquots of molten top agar, mixed and quickly poured and distributed over the surface of minimal glucose plates. Plates were protected from light and allowed to solidify. The plates were finally inverted and incubated at 370C for 48 hours. Following 48 hours incubation, the number of revertant colonies was determined per plate. Each test article concentration was analysed in triplicate in both the presence and absence of S9 enzyme activation system. In each of the two strains, negative control plates consisted of tester strain and S9 mix alone, (no test chemical) allowing identification and quantification of spontaneous bacterial revertants.
Positive controls included the diagnostic mutagens, 4-nitroquinoline-N-oxide (NQNO) for TA, 98 (250 µg / plate) and sodium azide for TA 100 (10 µg /

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plate) to demonstrate the reversion of each tester strain in the absence of metabolic activation.

Ames test for metal based drugs mutation assay:

In the Ames test all samples, 1,10-phenanthroline-5,6-dione, and metal complexes, were investigated. Each sample was investigated with the plate incorporation test in the absence and presence of S9 as described above.

Therefore 0.1 ml of the test compound in an appropriate concentration was added to 2-ml top agar and 0.5 ml S9. Three plates were incubated for each of the dilutions tested, except the solvent controls (negative control) where five plates were used. A positive control was also included (4-nitroquinoline-N-oxide (NQNO) for TA 98 and sodium azide for TA 100. A sample was considered mutagenic when there was a dose-dependent increase in revertants compared to the negative control. Dilutions that were investigated were MIC90, ½ MIC90 and ¼ MIC90 of each drug.

Results

MIC test:

Neutrient broth media was used for the anti-bacterial susceptibility testing. Neutrient broth (6.5 g) was dissolved in cold distilled water (500 ml) in 1L Duran bottle. Distributed to a final container, autoclaved and then allowed cooling. Prior to MIC testing, cells were grown on the same media at 30 ^{0}C 200 rpm for 24 h. At 650-nm absorbance was measured. A microtitre plate was inoculated with cells at density of 0.05 absorbance. Solution of the test drugs were prepared by dissolving the solid (0.02 g) in 500µl DMSO completed to 10 ml with distilled water to yield a stock solution of concentration 2000 µg/ml. One ml of stock solution and 9 ml of NB medium was used to yield drug solution of 200µg/ml final stock solution. One hundred µl of stock solution was transferred to the third column of microtiter plate. 50:50 serial dilutions were made to give dilution range (50 µg/ml to 0.1 µg/ml). The drug/cells mixtures were incubated at 30 ^{0}C for 24 h static incubator and the assay were performed in triplicate. Plates were read using RMX plate reader USA and data were statistically analysed using Microsoft Excel.

Determination of MIC_{90} of antibiotics & MBDs against both types of bacteria:

This experiment sought to determine the concentration of different MBDs that inhibited the growth of Gram positive and Gram negative bacteria. Metal salt, ligand phendio and metal complexes were assayed against gram-positive and gram-negative bacteria in order to determine the MIC_{90} and to detect if the spectrum of activity of the complex was changed with respect to that of the metal salt and ligand phendio. Seven types of bacteria were used for test experiment. The result in Table (1) shows the results of the *in vitro* antimicrobial activity of the compounds. The metal complexes shows the different minimal inhibitory concentration to the corresponding metal salt and phendio.

Antibiotics	*Staphylococcus aureus*	*Micrococcus luteus*	*Staphylococcus Simulanes*	*MRSA*	*Escherichia coli*	*Bacillus oleronius*	*Pantonia agglumerus*
Gentamicin	0.29 ± 0.2	0.31 ± 0.42	0.37 ± 0.3	0.38 ± 0.1	0.41 ± 0.2	0.49 ± 0.3	0.51 ± 0.2
Tetracyclin	22.3 ± 1.9	38.7 ± 2.1	41.1 ± 3.6	44.13 ± 4.1	12.9 ± 1.8	15.1 ± 1.7	16.3 ± 2.2
Lincomicin	41.1 ± 3.9	48.1 ± 3.9	37 ± 3.1	>50	>50	39.5 ± 5.1	43.1 ± 3.6
Rifampicin	7.6 ± 1.3	10.7 ± 2.1	9.2 ± 1.8	11.1 ± 2.0	6.1 ± .4	5.9 ± 1.1	6.9 ± 2.0
Chloramphenicol	1.9 ± 0.3	2.1 ± 0.4	2.0 ± 0,8	1.2 ± 0.3	4.8 ± 2	6.1 ± 1.1	9.4 ± 1.3
1,10-Phenanthrolin-5,6-dione	1.1 ± 0.4	3.8 ± 0.7	1.0 ± 0.4	0.2 ± 0.2	0.6 ± 0.2	0.2 ± 0.1	1.8 ± 0.6
$Ag(ClO4)_2$	8.4 ± 1.0	4.9 ± 2.1	12.1 ± 0.5	0.8 ± 0.5	10.7 ± 1.7	1.0 ± 0.9	11.2 ± 1.3
$[Ag(phendion)_2]ClO4$	3.8 ± 1.7	2.0 ± 0.9	1.9 ± 0.8	0.7 ± 0.7	2.0 ± 0.9	1.6 ± 1.2	2.1 ± 1.4
$[Cu(ClO4)_2].6H2O$	>50	>50	>50	>50	>50	>50	>50
$[Cu(phendion)_3(ClO4)_2.4H_2O$	1.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	1.0 ± 0.4	2.0 ± 1.4	1.2 ± 0.3

Table 1: MIC ug/ml value of some currently used antibiotics and metal based drugs

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All the drugs tested have antibacterial activity except the $Cu(ClO_4)_2.6H_2O$ which show the MIC more than 50 µg/ml for all strains. $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ show the lower MIC_{90} than the metal salts and phendio against *Staphylococcus simulanes*, *Pantonea agglumerans* and *Micrococcus luteus*, and higher MIC_{90} against all other strains than that of phendio. $[Ag(phendio)_2]ClO_4$ and $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ show lower MIC_{90} than that organic phendio against the *Micrococcus luteus*. 1,10-phenanthroline-5,6-dione, $[Ag(phendio)_2]ClO_4$ and $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ have MIC_{90} between 0.2 to 3.9 µg/ml. The $Ag(ClO_4)_2$ show MIC_{90} between 1 and 5 µg/ml against the *Bacillus oleronius*, *Micrococcus luteus* and *MRSA* and more than 5 µg/ml against *S. simulans*, *S. aureus*, *Pantonia agglumerans* and *E. coli*.

Viability Assay of bacteria strain in presence of MBDs

Effect of metal based drugs on viability of experimental bacteria strains:

Bacteria cells were grown in nutrient broth in the absence of drugs to the late exponential phase (18-24 hr) at 30 ^{o}C 200rpm, absorbance was measured, and drugs were added to the culture at sub MIC_{90} levels in nutrient broth medium. Four hour further incubation at same condition with drugs, serial dilution of the culture in sterile PBS were made and 0.1 ml was spread-out onto nutrient agar plates. All Plates were incubated at 30 ^{0}C for 48hr, the colony number was counted and the result was expressed as a percentage with respect to the control. The data presented in figures 1, 2 show the effect of MBDs on viability of Gram negative bacteria, and figure 3, 4, 5, 6 show the effect of MBDs on viability of Gram positive bacteria.

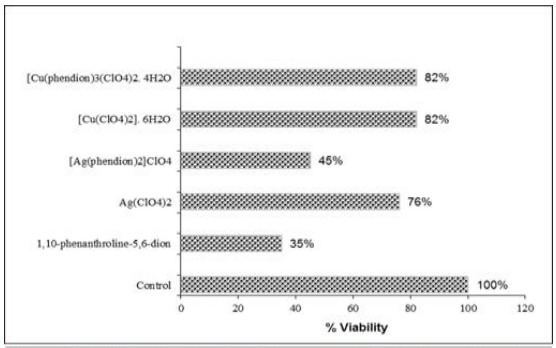

Figure 1: Viability of *Escherichia coli* when exposed for 4 hrs to metal based drugs.

The percentage of viability of all test bacteria in presence of sub MIC_{90} dose of $[Ag(phendio)_2]ClO_4$ was between 40 – 50 % except the *MRSA* and *Staph. simulans* where it was more than 70%. 1,10-phenanthrolin-5,6-dione has an inhibitory effect on the viability of *Staph. aureus* and *E. coli,* the percentage of viability was less than 35% and for other strains was more than 60% in respect to the control cells.

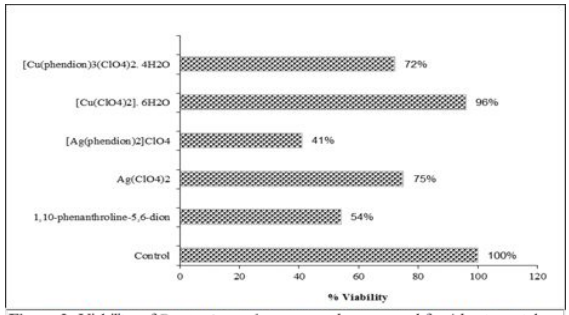

Figure 2: Viability of *Pantonia agglumerans* when exposed for 4 hrs to metal based drugs.

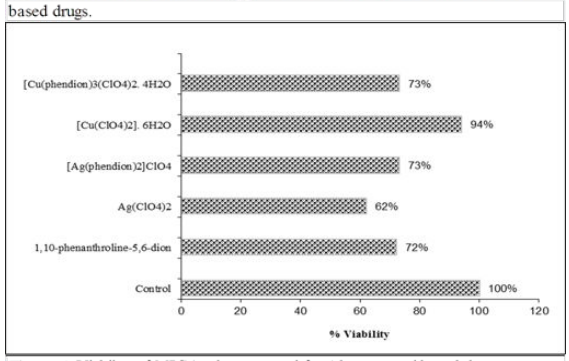

Figure 4: Viabilty of *MRSA* when exposed for 4 hrs to metal based drugs.

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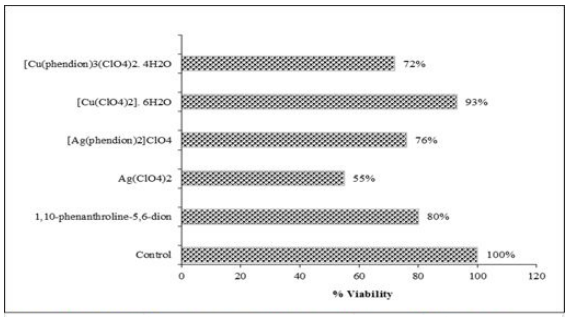

Figure 5: Viability of *Staphylococcus simulanes* when exposed for 4 hrs to metal based drugs.

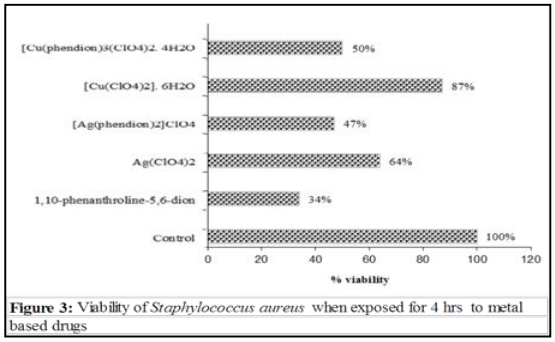

Figure 3: Viability of *Staphylococcus aureus* when exposed for 4 hrs to metal based drugs

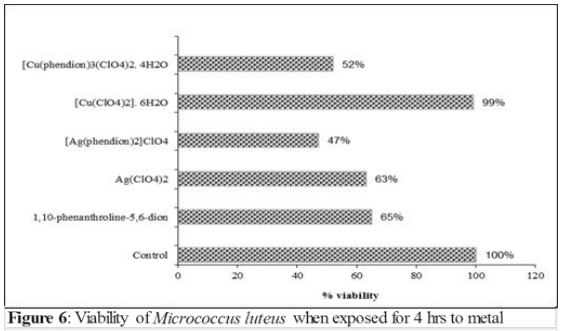

Figure 6: Viability of *Micrococcus luteus* when exposed for 4 hrs to metal based drugs.

Susceptibility Assay

Effect of MBDs on Susceptibility of bacteria to antibiotics:

Antibiotics susceptibility testing was performed using stationary phase cultures of bacteria that had been grown in nutrient medium (Sigma Aldrich) overnight as a control, all other samples were supplemented with ½ MIC_{90} of MBDs, incubation at 30 C 200 rpm for 24 h, cells (0.05) absorbance was tested using 96 well microdilution plate, all the antibiotic used were dissolved in DMSO and diluted with the nutrient broth media to final concentration. (100µl) was added to all wells on a 96 well plate with an extra addition of 100µl to lane number one, which was used as the blank (control media). One hundred microlitres of the highest final concentration of Antibiotics (20µg/ml) was added to lane 3 and 100µl was then transferred to lane 4 and from there after, a 50:50 series of dilutions of Antibiotics were done all the way to lane 12. Nutrient broth (100µl) with cells pre-grow in the presence of sub MIC_{90} of MBDs were then added from lane 2 to lane 12. Lane 2 was the positive control (medium, bacteria and MBDs) on each plate. The objective of this test was to provide the lowest concentration of antibiotics necessary to inhibit growth by 90% (MIC_{90}) compared to the positive control. A control 96-well plate was also done whereby certain type of bacteria was exposed to the range of dilutions of antibiotic tested without any MBDs. Each plate was put under static conditions at 30°C and the optical density was read at 450 nm using a MRX spectrophotometer (Dynax Technology, Chantilly, VA, USA). The bacteria used in this study were *Staph. aureus* and *E. coli*.

Table 2 show the effect of MBDs on the susceptibility of *bacteria* to antibiotics listed.

Antibacterial Agent		MIC90	
		Staphylococcus aureus	***Escherichia coli***
Rifampicin (alone)		8	7
Culture suplemented with	1,10-Phenanthrolin-5,6-dione	4	4.2
	$Ag(ClO4)_2$	4.1	5.2
	$[Ag(phendion)_2]ClO4$	3.1	2.9
	$[Cu(ClO4)_2]$. 6H2O	8.1	7.2
	$[Cu(phendion)_3(ClO4)_2$. $4H_2O$	3.7	3.9
Antibacterial Agent		**MIC90**	
		Staphylococcus aureus	***Escherichia coli***
Lincomicin (alone)		20	> 50
Culture suplemented with	1,10-Phenanthrolin-5,6-dione	9.8	36.4
	$Ag(ClO4)_2$	7.1	29.9
	$[Ag(phendion)_2]ClO4$	7	18.9
	$[Cu(ClO4)_2]$. 6H2O	18.2	> 50
	$[Cu(phendion)_3(ClO4)_2$. $4H_2O$	17.9	> 50

Table 2: Effect of MBDs on susceptibility of *Staphylococcus aureus* and *Escherichia coli* to Rifampicin and Lincomicin

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When culture pre-grown in sub-MIC_{90} of $[Ag(phendio)_2]ClO_4$ and $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ the susceptibility of *E. coli* to Rifampicin was increased.. The MIC_{90} of Rifampicin was 7, 6 µg/ml but when the bacterial cells were pre-grown in sub MIC_{90} of phen-dione and metal complex the sensitivity of cells increased leading to MIC_{90} of less than 4 µg/ml. Similar results were found with susceptibility assay of *Staph. aureus* to Lincomycin. When the cells pregrown in sub MIC_{90} of $Ag(ClO_4)_2$ and $[Ag(phendio)_2]ClO_4$. The MIC_{90} of Lincomycin was 20 µg/ml but when the cells were treated with these compounds the inhibitory effect of Lincomycin was increased and the MIC_{90} dropped to 7 µg/ml. On other hand the $Cu(ClO_4)_2.\ 6H_2O$ and $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ has no effect on susceptibility of *Staph aureus* to Lincomycin.

Mutagenicity test

All compounds tested did not appear mutagenic at all dilutions and there is no dose dependent increase according to the result shown in Table 3. The Figure 7, 8, 9, 10 show the average number of revertant colonies of *S. thyphimurium* TA98 and TA100 in presence and absence of S9 (rat liver enzymes). In Figure 7 show the mutagenic test of MBDs on test strains of *Salmonella typhimurium* TA98 in absence of S9. The average number of revertant colonies in the positive control was 214 colonies per plate; all test compounds are values between 24 to 50 colonies per plate.

Table 3: result of mutagenic assay show the revertant colonies ±SD of TA98 and TA100 in present and absent of S9, metal based drugs tested in serial dilution

Salmonella typhimurium strain TA98 Positive S9

1,10-Phenanthrolin-5,6-dione			[Ag(phendion)$_2$]ClO4			[Cu(phendion)$_3$(ClO4)$_2$. 4H$_2$O		
Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD
0	39	± 3	0	39	± 3	0	39	± 3
1/4 MIC_{90}	42	± 5	1/4 MIC_{90}	38	± 11	1/4 MIC_{90}	43	± 7
1/2 MIC_{90}	38	±10	1/2 MIC_{90}	32	± 3	1/2 MIC_{90}	31	± 6
MIC_{90}	45	± 7	MIC_{90}	36	± 9	MIC_{90}	39	± 9

Salmonella typhimurium strain TA98 negative S9

1,10-Phenanthrolin-5,6-dione			[Ag(phendion)$_2$]ClO4			[Cu(phendion)$_3$(ClO4)$_2$. 4H$_2$O		
Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD
0	32	± 4	0	32	± 4	0	32	± 4
1/4 MIC_{90}	38	± 11	1/4 MIC_{90}	41	± 6	1/4 MIC_{90}	35	± 7
1/2 MIC_{90}	41	± 21	1/2 MIC_{90}	36	± 12	1/2 MIC_{90}	27	± 12
MIC_{90}	45	± 7	MIC_{90}	36	± 9	MIC_{90}	33	± 9

Salmonella typhimurium strain TA100 Positive S9

1,10-Phenanthrolin-5,6-dione			[Ag(phendion)$_2$]ClO4			[Cu(phendion)$_3$(ClO4)$_2$. 4H$_2$O		
Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD
0	93	± 15	0	93	± 15	0	93	± 15
1/4 MIC_{90}	52	± 13	1/4 MIC_{90}	67	± 19	1/4 MIC_{90}	81	± 17
1/2 MIC_{90}	75	± 22	1/2 MIC_{90}	48	± 13	1/2 MIC_{90}	55	± 20
MIC_{90}	45	± 14	MIC_{90}	52	± 11	MIC_{90}	59	± 11

Salmonella typhimurium strain TA100 Negative S9

1,10-Phenanthrolin-5,6-dione			[Ag(phendion)$_2$]ClO4			[Cu(phendion)$_3$(ClO4)$_2$. 4H$_2$O		
Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD	Dose	Revertant colonies	± SD
0	71	±31	0	71	± 31	0	71	± 31
1/4 MIC_{90}	73	± 12	1/4 MIC_{90}	59	± 12	1/4 MIC_{90}	69	± 7
1/2 MIC_{90}	67	± 19	1/2 MIC_{90}	69	± 22	1/2 MIC_{90}	67	± 27
MIC_{90}	69	± 9	MIC_{90}	63	± 8	MIC_{90}	72	± 5

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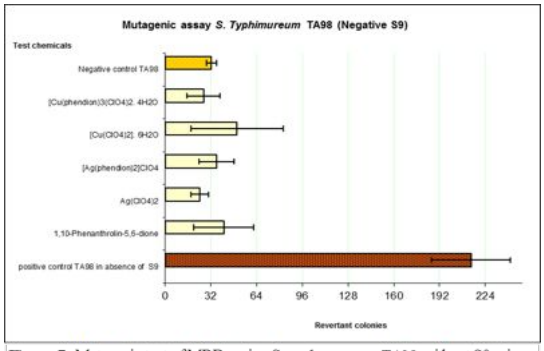

Figure 7: Mutagenic test of MBDs using *S. typhimureum TA98* without S9 mix

In presence of metabolic enzyme S9 as shown in Figure 8 the TA98 colonies for the positive control was 235 colonies per plate and all compounds gave 25 to 40 colonies per plate. The result of tester strains *Salmonella typhimurium* TA100 was shown in Figure 9 and 10 in presence and absence of S9 respectively.

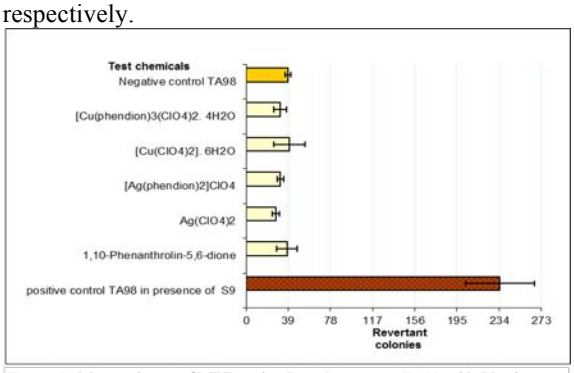

Figure 8: Mutagenic test of MBDs using *S. typhimureum TA98* with S9 mix

In absence of S9 the positive control was 1095 colonies per plate and in presence of S9 the positive control was 1008 colonies per plate. In both tests the result of revertant colony was in the range of the negative control limit.

The revertant colonies of both strains were under the mutagenic range as appear in graphs according to positive and negative controls (figure, 11).

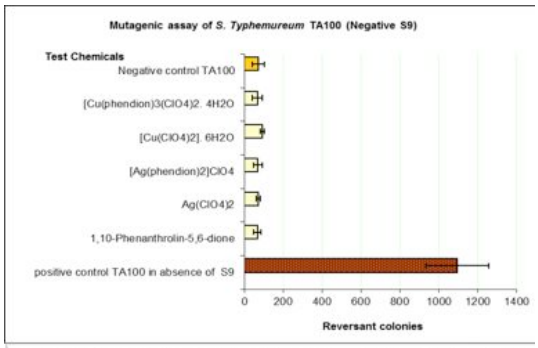

Figure 9: Mutagenic test of MBDs using *S. typhimureum TA100* without S9 mix

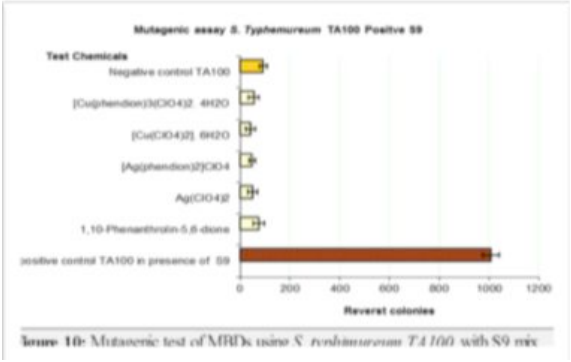

Figure 10: Mutagenic test of MBDs using *S. typhimureum TA100* with S9 mix

Discussion:

There are many different mechanisms of antimicrobial action, which are operative in destroying bacteria, fungi and yeast. Each mechanism has a specific target and the resulting activity in case of bacteria can be bacteriostatic or bactericidal. Most antibiotics are given as single agents. There are cases however, when two drugs are used in combination. Antimicrobial agents may affect each other when used in combination. The effect may simply be additive. In some cases the activity of one drug enhances that of a second drug. This is referred to as synergy. Alternatively, drugs may interfere with each other this is called antagonism. Penicillins and bacteriostatic drugs such as tetracyclines are antagonistic, since penicillins require actively growing cells and static drugs prevent cell growth. In contrast, aminoglycosides are synergistic when used in combination with penicillins (7). Previous studies showed some variation of activity against Gram-negative organisms to include *P. aeruginosa* as well as the Gram-positive *S. aureus* (8). The present study revealed that combining MBDs with some currently used antibiotics produces altered efficacy on some Gram negative and Gram positive bacteria which remained totally susceptible, as did the *C. albicans.* Such a result was seen when *Staphylococcus aureus* and *E coli* were tested with Lincomycin and Rifampicin respectively. Based on these data we

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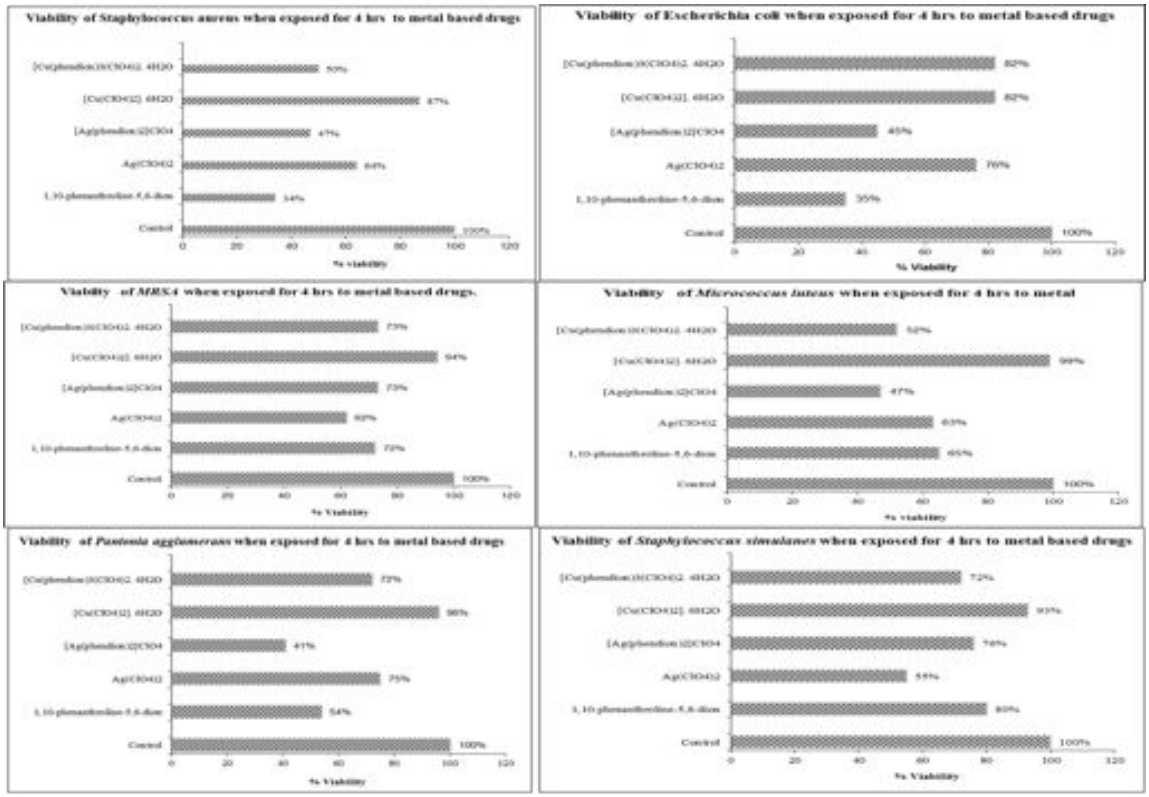

Figure 11: Effect of MBDs on Bacteria viability

conclude that combining MBDs particularly $[Ag(phendio)_2]ClO_4$ and $[Cu(phendio)_3](ClO_4)_2{\cdot}4H_2O$ with antibiotic is effective *in vitro* in some cases specifically when Rifampicin is used against Gram-negative bacteria, particularly *Escherichia coli*. We therefore determined the *in vitro* susceptibility of Gram negative and Gram-positive bacteria to various antibiotics and their combinations with MBDs and also measured the alteration of the susceptibility spectrum of our bacterial strains and the variations of bactericidal effect of these antibiotics. Several studies have previously reported synergistic effects between antibiotics. The work presented here demonstrates that using sub-MIC_{90} level of MBDs it is possible to alter the amount of antibiotic required to achieve 90 percent inhibition of growth of different types of bacteria. Such a situation would reduce the amount of conventional drugs needed along with MBDs to get the same response, as would be achieved using high dose of antibiotics alone.

Newly discovered products (pharmaceuticals, foods and food additives, and other chemicals) need a thorough investigation of their safety and efficacy to human health before release onto the market. Most regulatory agencies require that the drugs undergo a series of toxicological tests, including mutagenesis testing. Genetic toxicology is concerned primarily with the mutational effects of chemicals, where a mutation is defined as a permanent change in the amount or structure of the genetic material in a cell. Mutagenicity tests are used to screen newly synthesised chemicals for the ability to induce mutations. It is now clear that DNA is the basic carrier of genetic information common to all living cells and that damage to DNA is the fundamental mechanism of induced mutation (10). Many tests used for the cytotoxicity of chemical compounds include tests such as interaction of chemical and DNA, DNA damage and repair, Ames test (Gene mutation) and Chromosome aberration assay (DNA breakage). In order to test for gene mutations in bacteria Ames Test was used in this study because it is the most widely used test for assessing the

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mutagenic properties of chemicals. The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay that uses a set of histidine-requiring strains of bacteria to detect frameshifts and base pair substitution mutations (11). Treatment with mutagens can induce the mutations in the histidine operon and shift growth of the strains from a histidine-requiring to a histidine-independent pattern (12). The change in the growth phenotype represents an indicator of mutagenic response. The role of metabolic activation on the mutagenic effect of chemicals, was assessed by using metabolic activation fraction of rat liver homogenate enzyme (S9) to detect the mutagenicity effect of drug metabolites. Metal based drugs were tested for their cytotoxicity since these have been reported in a number of publications as antifungal agents (1), antibacterial agents (7) and alter the susceptibility of *C. albicans* to polyene and azole drugs (9). For the safe use of antifungal and antibacterial drugs Ames Test was used to assess the drug and drug metabolite by testing the drugs in absence and presence of liver metabolic enzyme system. All the results using test bacteria (TA98 and TA100) give indication of no mutagenic effect of tested compounds. The *in vitro* result of test give an indication of harmless and safety of use according to Ames Test. The metal based drugs were not mutagenic. Other assays such as Genetic *in vivo* Assays are recommended to use to give confirmation of drug safety for human use.

In conclusion, this study shows that phendio, metal salt and metal complexes have antibacterial activity by inhibit bacteria growth and viability. Bacteria pre-grown in sub-MIC_{90} overnight and then assessed for susceptibility to some currently used antibiotics give high bactericidal rate than when the antibiotic is used alone. Silver and copper phendio complexes might represent potential candidates for combination treatments with standard antibiotics to enhance anti-bacterial efficacy of drugs such as Lincomycin and Rifampicin against *S. aureus* and *E. coli*, respectively.

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