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Evaluation of lipophilicity, antimicrobial activity and mutagenicity of some novel ester prodrugs of metronidazole

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Various novel aliphatic and aromatic esters of metronidazole have been synthesized to improve the physicochemical properties (Rm values, lipophilicity) using prodrug approach. The alcoholic functional group of metronidazole is readily esterified, thus several novel ester prodrugs of metronidazole have been synthesized and evaluated for their anaerobic antibacterial activity and mutagenicity. These compounds have been characterized by UV, IR, ^{1}H NMR, mass spectra and elemental analysis. The partition coefficient of esters is determined by *n*-octanol/water system, RP-TLC and computed *in silico*. Anaerobic activity against *C. perfringens*, determined in terms of MIC (μg/mL) show the esters, particularly SDS-18 and SDS-19, to be more potent in comparison to metronidazole. The Ames test is used to compare the mutagenic potential of the synthesized 5-nitroimidazoles.

Keywords: Prodrug, 5-nitroimidazole, lipophilicity, antibacterial activity, anaerobic, Ames testing

Metronidazole, a 5-nitroimidazole possess potent anti-protozoal and antibacterial activity and has gained wide acceptance for the systemic treatment of trichomoniasis, giardiasis, amoebiasis, acute ulcerative gingivitis and dental infection inspite of being a bacterial mutagen and rodent carcinogen[1-3]. It is also having undesirable properties such as poor absorption; low aqueous solubility and unwanted side effects, which have direct influence on its pharmacological and pharmacokinetic properties[4]. By utilizing the alcoholic functional group of metronidazole several novel ester prodrugs of metronidazole were synthesized with an aim to increase lipid solubility, so that it can be used to make injectible formulation.

In general, all ester of metronidazole were prepared by first converting the acid (propionic acid, butyric acid, valeric acid, caproic acid, benzoic acid or phenyl cinnamic acid) into their respective acid chlorides **(S1-S6)** using thionyl chloride under anhydrous condition (**Scheme I**). The acid chlorides were then made to react with –OH functionality of metronidazole to get different esters **(SDS-20 – SDS-25)** using dichloromethane as solvent in the presence of a base at room temperature The synthesis of metronidazole acetate {(2-(5-methyl-2-nitro-1*H*-imidazol-1yl)ethyl acetate} **(SDS-19)** was carried out by reacting acetic anhydride with metronidazole **1** in the presence of pyridine under anhydrous condition **(Scheme II)**. The synthesized compounds were characterized by TLC, melting/boiling point, UV, IR, ^{1}H and ^{13}C NMR, CHN analysis and mass spectroscopy.

Results and Discussion

Partition coefficient

The log P of all the synthesized derivatives and standard metronidazole (MTZ) was calculated experimentally using shake flask method (*n*-octanol/water system) and taking the absorbance of the samples in UV spectrophotometer at their respective λ_{max}. The log P values were also predicted *in silico* using Chembridge® software.

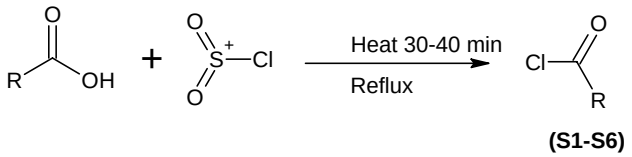
The lipophilicity of the synthesized compounds and metronidazole were also determined by RP-TLC method (which is a well accepted method for the determination of lipophilicity within a homologous series)[5,6]. Their R_f values were first determined using impregnated silica gel plates as stationary phases and mixtures of aqueous phosphate buffer (*p*H= 7.4) in methanol (50-90%, v/v) as eluent. The obtained R_f values were used in calculation of the corresponding Rm values according to the following equation:

$$R_m = \log \{1 / R_f - 1\} \quad \ldots(1)$$

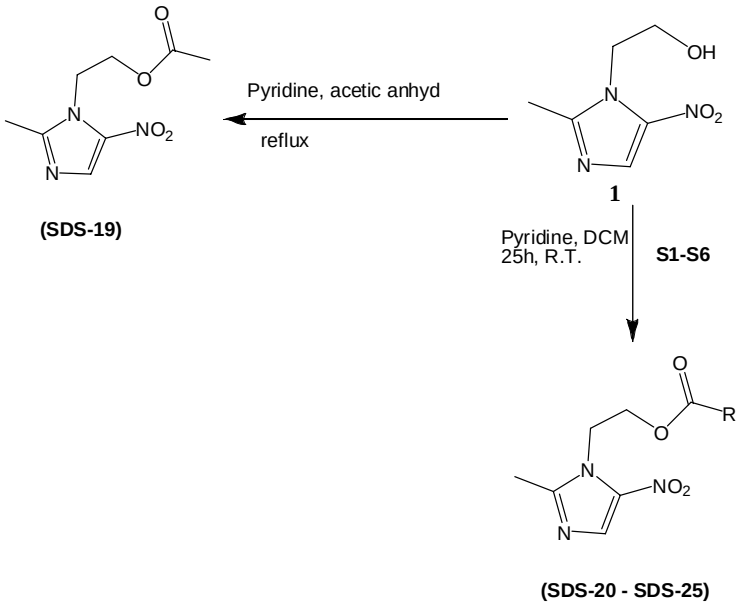
The results are presented in **Table I**. The R_m values at 0% methanol were then calculated from the regression derived by correlating the calculated R_m values of synthesized compounds and metronidazole to the respective percentage of methanol. A comparison of *in silico* prediction and experimentally calculated log P values along with the Rm values are given in **Table II**. The results show that there is an increase in the lipophilicity of the synthesized ester derivative with increase in carbon chain length of acyl group when compared with metronidazole. This can lead to an increase in the transport across the biological membrane and into the cells by passive diffusion.

Antimicrobial activity

The anaerobic antibacterial activity is determined in terms of MIC in μg/mL by using serial dilution method[7,8] against *Clostridium perfrigens* (ATCC-11437). The result of MIC of the standard drug metronidazole and its synthesized esters derivative are given in **Table III**. The MIC values of all esters against *C. perfrigens* were low in comparison to metronidazole. The esters **SDS-23** and **SDS-24** show significantly more activity than metronidazole and other compounds. The compound **SDS-25** has comparable activity to metronidazole. As the carbon chain of esters increases, the antibacterial activity also increases. The compound **SDS-24** is most active of all the synthesized prodrug derivatives suggesting that the antimicrobial activity increases with increase in the carbon chain length of the acid. The antimicrobial (anaerobic) activity is following the order **SDS-24** > **SDS-23** > **SDS-22** > **SDS-21** > **SDS-20** > **SDS-19** > **SDS-25** > **1**. This order of activity suggests that as the lipophilic character (as per experimentally calculated log P values) increases from two-carbon chain length to six-carbon chain length and even more on cyclization/aromatization of the side chain; this leads to an increase in the anti microbial property. But further increase in lipophilic character following introduction of biphenyl ring system as in **SDS-25**,



Scheme I



R		**R**	
SDS-20	$-CH_2CH_3$	**SDS-24**	$-C_6H_6$
SDS-21	$-CH_2CH_2CH_3$		
SDS-22	$-CH_2CH_2CH_2CH_3$	**SDS-25**	
SDS-23	$-CH_2CH_2CH_2CH_2CH_3$		

Scheme II

Table I— Rm values of the metronidazole esters (SDS-19-SDS-25) and metronidazole **1**

Compd	% of methanol in phosphate buffer (v/v)							R^2
	10%	15%	20%	30%	40%	50%	0%	
1	-0.035	-0.126	-0.175	---	-0.228	-0.328	-0.0149	0.9057
SDS-19	-0.593	---	-1.08	-1.62	-2.101	---	-0.0825	0.9995
SDS-20	0.379	---	---	0.1799	0.112	---	0.465	0.9926
SDS-21	0.727	---	0.647	0.56	0.473	0.345	0.8298	0.9922
SDS-22	0.546	0.421	---	0.248	0.149	0.035	0.6331	0.9847
SDS-23	---	0.417	0.249	0.09	-0.025	-0.147	0.5901	0.9652
SDS-24	0.93	0.825	0.687	0.585	0.425	---	1.0644	0.9762
SDS-25	---	1.128	1.06	0.815	0.569	---	1.4935	0.9937

Table II — Computed, experimentally determined log P values and Rm value at 0% methanolic concentration of metronidazole and synthesized compounds

Compd	Rm value at 0% methanolic concentration	Experimental log P values	Software calculated log P values
1	-0.0149	0.7512	-0.013
SDS-19	-0.0825	1.0123	0.47
SDS-20	0.465	1.3518	1.01
SDS-21	0.8298	2.085	1.54
SDS-22	0.6331	2.0145	2.07
SDS-23	0.5901	2.011	2.6
SDS-24	1.0644	2.65	2.5
SDS-25	1.4935	3.998	4.14

Table III — *In vitro* antimicrobial activity of the synthesized compound and metronidazole against *Clostridium perfrigens* ATCC-11437

Compd	MIC (Serial dilution assay) (μg/mL)
1	9
SDS-19	7
SDS-20	6.5
SDS-21	3
SDS-22	2.5
SDS-23	0.8
SDS-24	0.4
SDS-25	7.5

(increasing the bulk), tends to lower down the antimicrobial activity significantly.

Mutagenicity testing

The mutagenicity activity was carried out by Ames salmonella mutagenicity assay using TA-100 strain of *S. typhimurium* at dose level of 0.5 μmole, 1 μmole and 5 μmoles. Mutant strain of the bacterium *Salmonella typhimurium* (TA-100), are dependent on the histidine for the growth. These strains are applied to plate containing small amount of histidine and the test compound[9]. A compound is considered to be mutagenic if there is a statistically significant dose related increase in the number of revertants colonies[10]. The compounds were found to be active mutagens up to 1 μmole dose level beyond, which they were inhibitory. From the **Table IV** the tested compound **SDS-23** and **SDS-24** showed maximum formation of revertants colonies when compared with metronidazole it means it is more mutagenic than metronidazole. Compound **SDS-19, SDS-20, SDS-21, SDS-22** and **SDS-25** showed less number of colonies thus less mutagenic potential than standard compound. The order of mutagenic activity is **SDS-23**> **SDS-24** > **1** > **SDS-25** > **SDS-21** > **SDS-19** > **SDS-22** >**SDS-20**.

Experimental Section

Melting points were determined in open capillaries and are reported uncorrected. The IR spectra were recorded on a Jasco FT IR-460 plus Fourier transform Infrared spectrometer. ^{1}H NMR spectra were scanned on a Bruker ultraspec 500 MHz/ AMX 400 MHz spectrometer using $CDCl_3$ as solvent (chemical shift in δ ppm). The mass spectra were recorded on LCMS-2010A (Shimadzu) LC-Mass technique. Elemental analysis was performed in Thermo Finnigan CHN analyzer.

Synthesis of 2-(5-methyl-2-nitro-1*H*-imidazol-1yl)-ethyl acetate (SDS-19)

A mixture of acetic anhydride (5 mL, 0.052 *M*) and metronidazole (500 mg, 2 m*M*) in pyridine (5 mL) was heated on a water bath for 2 hr. After this period solution was cooled at room temperature and added to

Table IV — Ames Salmonella mutagenicity assay of the compounds **SDS-19** to **SDS-25** and metronidazole

Compd Dose (m*M*)	Revertants/Plate							
	MTZ	19	20	21	22	23	24	25
0.5	192	73	26	16	56	196	101	82
	190	45	33	22	38	190	100	80
	187	72	27	20	56	195	102	69
1	213	74	57	115	70	225	215	129
	180	75	48	110	55	220	225	126
	200	85	55	122	58	228	218	138
5	0	0	0	0	13	16	22	0
	0	0	0	0	12	14	26	0
	0	0	0	0	16	13	24	0
Mean	193.66	70.660	41.000	67.5	41.55	144.11	114.77	104
SD	11.500	13.462	14.042	52.936	10.232	17.111	65.898	30.166
SEM	64.708	24.009	15.603	35.802	18.771	70.404	63.283	38.010
Variance	3.39	3.66	3.74	7.27	3.19	4.13	8.11	5.492

ice cold water. The separated compound was extracted with chloroform (25 mL) three times. The combined chloroform extracts was washed with 1M sodium carbonate (25 mL) followed by washing with 10% HCl (25 mL). Then chloroform layer was washed three times with distilled water. The water layer was discarded and chloroform layer was dried over anhydrous sodium sulphate for about half an hour and the solvent was removed *in vacuo*. The solid mass was recrytallised with chloroform to yield the product. m.p. 70-71ºC, yield 86%, R_f value = 0.8. UV λmax (MeOH): 318.5 nm; IR (KBr): 3123.15 (Ar-CH), 2993.94(Al-CH), 1740.49 (C=O), 1697.05 (C=N), 1568.81 (N=O), 1464.67(C=C), 1395 (C-NO_2) and 1061.25 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.05 (m, 2H, -OCC*H*$_3$), 2.52 (s, 3H, C*H*$_3$ group, imidazole), 4.4 (t, 2H, >N-C*H*$_2$); 4.58 (t, 2H, C*H*$_2$-O), 7.96 (s, 1H, imidazole ring *H*); ^{13}C NMR ($CDCl_3$): δ 13.23, 19.52, 43.97, 61.44, 132.04, 137.43, 149.85, 169.13; LC-MS: *m/z* 214 (M+1), 191, 160, 119, 103, 87. Anal. Calcd for $C_8H_{11}O_4N_3$: C, 45.07; H, 5.20; N, 19.71. Found: C, 45.57; H, 5.09; N, 19.85%.

General method for the synthesis of different metronidazole esters (SDS-20 – SDS-25)

Synthesis of different acyl chlorides (S1-S6)

A 100 mL two-necked flask was fitted with a dropping funnel and a reflux condenser connected at the top to a gas adsorption trap. A 10 mL redistilled thionyl chloride (0.137 *M*) was placed in the flask and 10 mL acid (propionic acid, butyric acid, valeric acid, caproic acid, benzoic acid and phenyl cinnamic acid) (0.133 *M*) in a separating funnel. The flask was heated gently on a water bath, and acid was added during the course of 30-40 min. After adding the acid, the reaction mixture was heated on a water bath for 30 min. The excess thionyl chloride was removed *in vacuo* to give different acyl chloride, which was immediately used for next step.

Synthesis of different metronidazole esters (SDS-20 – SDS-25)

A solution of different acyl chloride in methylene chloride (10 mL) was added drop-wise to a mixture of metronidazole (1 g, 5.8 m*M*), pyridine (1 mL) and methylene chloride (50 mL) under anhydrous condition. The resulting solution was stirred at room temperature for 25 hr. The solvent was removed *in vacuo* and the residue obtained was stirred with 1*M* sodium carbonate solution (25 mL) for 15 minute. It was followed by extraction of the material with chloroform (3 × 25 mL). The chloroform extract was washed with 10% HCl (25 mL) and then with distilled water (3 × 25 mL). The chloroform extract was dried over anhydrous sodium sulphate and solvent removed *in vacuo* to give different metronidazole esters.

2-(5-methyl-2-nitro-1*H*-imidazol-1-yl)ethyl propionate (SDS-20)

Semi solid, Yield 79.1%, b.p. 119-120ºC, R_f value = 0.75; UV λmax (MeOH): 319.5 nm; IR (KBr): 3136.04 (C-H aromatic), 2985.6(C-H aliphatic), 1741.6 (C=O), 1654.81 (C=N), 1541.02 (N=O),

1429.15(C=C), 1400 (C-NO_2) and 1080 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.16 (m, 3H, -OCCH$_2$C*H*$_3$), 2.36 (s, 3H, -C*H*$_3$ group, imidazole), 2.55 **(**m, 2H, -OCC*H*$_2$), 4.43 (t, 2H, >N-C*H*$_2$), 4.62 (t, 2H, C*H*$_2$-O), 7.95 (s, 1H, imidazole ring *H*); ^{13}C NMR ($CDCl_3$): δ 8.96, 13.66,27.16, 44.98, 62.28, 131.58, 138.14, 150.95, 173.9; LC-MS: *m/z* 228 (M+1). Anal. Calcd for $C_9H_{13}O_4N_3$: C, 47.57; H, 5.77; N, 18.50. Found: C, 47.28; H, 4.99; N, 17.88%.

2-(5-Methyl-2-nitro-1*H*-imidazol-1-yl)ethyl butyrate (SDS-21)

Semi solid, Yield 89.1%, b.p. 115-116ºC, R_f value = 0.60; UV λmax (MeOH): 318.5 nm; IR (KBr): 3134.11 (C-H aromatic), 2968.24 (C-H aliphatic), 1739.67 (C=O), 1698.05 (C=N), 1529.45 (N=O), 1471.59(C=C), 1395 (C-NO_2) and 1095.49 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.0 (m, 3H, CH_2-C*H*$_3$), 1.71-1.57(m, 2H, -OCCH$_2$C*H*$_2$CH$_3$), 2.34-2.24 **(**m, 2H -OCC*H*$_2$CH$_2$CH$_3$), 2.55(s, 3H, C*H*$_3$ group, imidazole), 4.44 (t, 2H, >N-C*H*$_2$), 4.58 (t, 2H, C*H*$_2$-O), 7.95 (s, 1H, imidazoline ring *H*); ^{13}C NMR ($CDCl_3$): δ 13.12, 17.94, 35.66, 42.31, 47.47, 62.16, 130.8, 138.06, 150.69, 173.15; LC-MS: *m/z* 242 (M+1), 172, 125. Anal. Calcd for $C_{10}H_{15}O_4N_3$: C, 49.79; H, 6.27; N, 17.42. Found: C, 49.80; H, 5.99; N, 17.36%.

2-(5-Methyl-2-nitro-1*H*-imidazol-1-yl)ethyl pentanoate (SDS-22)

Semi solid, Yield 78.9%, b.p. 122-123ºC, R_f value = 0.88; UV λmax (MeOH): 318.5 nm; IR (KBr): 3132.18 (C-H aromatic), 2968.46(C-H aliphatic), 1745.46 (C=O), 1652.88 (C=N), 1531.37 (N=O), 1469.66(C=C), 1385 (C-NO_2) and 1037.63 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.84-0.82 (m, 3H,-CH$_2$CH$_2$C*H*$_3$), 1.32-1.16 (m, 2H, -CH$_2$C*H*$_2$CH$_3$), 1.55-1.42 (m, 3H, -C*H*$_2$CH$_2$CH$_3$), 2.26-2.1 **(**m, 2H, OCC*H*$_2$-), 2.44 (s, 3H, C*H*$_3$ group, imidazole), 4.31 (t, 2H, >N-C*H*$_2$), 4.50 (t, 2H, C*H*$_2$-O), 7.88 (s, 1H, imidazole ring *H*); ^{13}C NMR ($CDCl_3$): δ 13.84, 22.06, 26.70, 33.69, 45.03, 62.21, 131.53, 138.34, 150.79, 173.22, 178.97; LC-MS: *m/z* 256 (M+1), 172, 136, 125. Anal. Calcd for $C_{11}H_{17}O_4N_3$: C, 51.76; H, 6.71; N, 16.46. Found: C, 50.80; H, 6.14; N, 15.98%.

2-(5-Methyl-2-nitro-1*H*-imidazol-1-yl)ethyl hexanoate (SDS-23)

Semi solid, yield 79.1%, b.p. 142-43ºC, R_f value = 0.75; UV λmax (MeOH): 318.0 nm. IR (KBr): 3158.50 (C-H aromatic), 2933.53(C-H aliphatic), 1749.32 (C=O), 1652.88 (C=N), 1539.09 (N=O), 1463.87(C=C), 1390 (C-NO_2) and 1043.42 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.91 (m, 3H, -COCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_3$), 1.34-1.24 (m, 4H, -COCH$_2$CH$_2$C*H*$_2$C*H*$_2$CH$_3$), 1.67-1.54 (m, 2H, -COCH$_2$C*H*$_2$CH$_2$CH$_2$CH$_3$), 2.36-2.32 (m, 2H, OCC*H*$_2$), 2.57 (s, 3H, C*H*$_3$ group, imidazole), 4.41 (t, 2H, >N-C*H*$_2$), 4.60 (t, 2H, C*H*$_2$-O), 7.98 (s, 1H, imidazole ring *H*); ^{13}C NMR ($CDCl_3$): δ 13.32, 13.57, 22.15, 24.27, 31.09, 33.59, 33.86, 44.96, 62.16, 131.2, 173.15, 179.04; LC-MS: *m/z* 270 (M+1), 190,172. Anal. Calcd for $C_{12}H_{19}O_4N_3$: C, 53.52; H, 7.11; N, 15.60. Found: C, 52.81; H, 7.01; N, 15.28%.

2-(5-Methyl-2-nitro-1*H*-imidazol-1-yl)ethyl benzoate (SDS-24)

Solid, yield 85.12%, m.p. 84-85ºC, R_f value = 0.80; UV λmax (MeOH): 318.5 nm; IR (KBr): 3133.0 (C-H aromatic), 3032.51(C-H aliphatic), 1707.66 (C=O), 1603.52 (C=N), 1581.34 (N=O), 1499.38(C=C), 1380 (C-NO_2) and 1071.26 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.61 (s, 3H, C*H*$_3$ group, imidazole), 3.92 (t, 2H, >N-C*H*$_2$), 4.65 (t, 2H, C*H*$_2$-O), 7.26 (s, 1H, imidazole ring *H*), 8.22-7.47 (m, 4H, Ar-*H*); ^{13}C NMR ($CDCl_3$): δ 11.6, 40.8, 62.3, 120.6, 128.5, 129.9, 130.0, 130.1,138.5,155.2, 165.9; LC-MS: *m/z* 276 (M+1), 190. Anal. Calcd for $C_{13}H_{13}O_4N_3$: C, 56.72; H, 4.76; N, 15.27. Found: C, 56.8; H, 4.99; N, 15.88%.

2-(5-methyl-2-nitro-1*H*-imidazol-1-yl)ethyl 2,3-diphenyl acrylate (SDS-25)

Solid, yield 79.12%, m.p. 102-104ºC, R_f value = 0.80; UV λmax (MeOH): 318.5 nm; IR (KBr): 3051.8 (C-H aromatic), 3055.76(C-H aliphatic), 1749.12 (C=O), 1610.27 (C=N), 1570.74 (N=O), 1491.67(C=C), 1395 (C-NO_2) and 1069.33 (C-O-C) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.58 (s, 3H, C*H*$_3$ group, imidazole), 3.88. (t, 2H, >N-C*H*$_2$), 4.61 (t, 2H, C*H*$_2$-O), 7.37 (s, 1H, imidazole ring *H*), 7.26-7.0 (m, 11H, Ar-*H* and C=C*H*); ^{13}C NMR ($CDCl_3$): δ 76.83, 77.15, 77.47, 128.09, 128.32, 128.42, 128.79, 128.99, 129.51, 129.77, 130.03, 130.89, 131.09, 131.01, 134.41, 131.86, 134.01, 134.41, 134.87, 142.24, 144.06, 163.61; LC-MS: *m/z* 378 (M+1), 190. Anal. Calcd for $C_{21}H_{19}O_4N_3$: C, 66.83; H, 5.03; N, 11.13. Found: C, 66.81; H, 4.99; N, 10.88%.

Determination of log P value using shake flask method

A 10 mg of metronidazole and each derivative dissolved in minimal amount of methanol was shaken up with 10 mL of octanol and 10 mL water for 24 hr in rotary shaker (the octanol and water used in experiment were previously saturated with each other overnight). After 24 hr the water layer was separated and absorbance of water was taken in UV spectrophotometer at the respective λ max of each compound after appropriate dilution by using water with methanol as a blank for solvent correction. The standard plots of each derivatives (2-10 μg/mL) and parent drug (metronidazole) in water (by dissolving compounds in minimal amount of methanol) were plotted separately. The standard plot was used to calculate the unknown concentration of derivative in the experiment and partition coefficient of synthesized compound by using equation :

$$\log P = \log 10 \text{ [Conc. in octanol] / [Conc. in water]} \quad \ldots(2)$$

Determination of R_m values

Silica gel G60 F254 plates (10 × 15 cm) were impregnated for 5 hr in 3% v/v octanol in acetone and air-dried overnight. Solution of the tested compounds and metronidazole in acetone (1 mg/mL) were spotted at 1.5 cm intervals and plates were then developed in jars previously saturated with the solvent system for one hour, using aqueous phosphate buffer (*p*H 7.4) in methanol (50-90%) to a solvent front of 12 cm height. The developed plates were air-dried and spots were visualized in iodine chamber and in UV-light at 254 nm. Triplicate experiments were carried out to ensure reproducibility of the results. The R_f values were determined and its mean values were used in calculation of corresponding R_m values by equation (1).

Ames Mutagenicity test

A 0.5 mM L-histidine/0.5 mM biotin was added to the top agar and 2 mL portion were distributed into sterile tubes maintained at 45ºC in a thermostatic water-bath. Preincubation test procedure was followed throughout to increase the sensitivity of test. Phosphate buffer *p*H 7.4 (0.5 mL) was transferred to sterile tubes. To it, was added 0.1 mL of bacterial culture (grown for about 10 hr in oxoid nutrient broth no. 2 at 37ºC with shaking) and 0.1 mL of the solution of test compound in water. The tubes were vortexed and incubated at 37ºC for 20 min. To these, 2 mL of top agar held at 45ºC was then added. The test compound were mixed by vortexing the contents for 3 sec. at a low speed and then poured into minimal glucose agar plates. The plates were quickly tilted and then placed covered on level surface to harden to get uniform distribution of top agar. Mixing, pouring and distribution was done within 20 sec in order to avoid stippled surface, which makes the scoring of revertants difficult within an hour, the plates were inverted and placed in a B.O.D. incubator at 37ºC. After 48 hr, the revertant colonies on test and control plates were counted and the presence of background lawn was confirmed in each case.

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