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Synthesis and *In Vitro* Biological Activity of Charge-Transfer Complexes of Stavudine and its Intermediates with Chloranilic and Picric Acids

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ABSTRACT: Charge-transfer complexes of stavudine and its intermediates with chloranilic and picric acids were synthesized in order to determine their antimicrobial and antioxidant activities to improve the efficacy. The purity of the synthesized complexes was judged by their elemental analyses, and their chemical structures were confirmed by UV-visible, FT-IR and ^{1}H NMR spectral studies. Complex, **1a** showed significant antimicrobial activity compared to standard drug. Complexes, **2a** and **2b** showed moderate antioxidant activity compared to standard drug by diphenylpicrylhydrazyl (DPPH) assay method.
KEY WORDS: Charge-transfer complexes, Stavudine, Electron acceptors, Antimicrobials, Antioxidant activity

INTRODUCTION

Diseases caused by microbial infection are a serious menace to the health of human beings and often have connection to some the other diseases. In order to combat these diseases, a number of drugs are available in clinical practice ranging from natural product antibacterial to tailor-made antibacterial drugs. Developing antimicrobial drugs and maintaining their potency in opposition to resistance by different classes of microorganisms as well as a broad spectrum of antibacterial activity are some of the major concern of research in this area. The enhanced prevalence of diseases caused by microorganisms has become a worldwide problem. Additionally, the development of resistance among pathogens to routinely used pesticides demands that a renewed effort should be made to seek antimicrobial agents which are effective against pathogenic microbes[1]. The health problem demands to search and synthesize a new class of antimicrobial compounds which are effective against pathogenic microorganisms and develop resistance to the antibiotics used in the current regime[2, 3]. The clinical relevance of fungal diseases has increased over the past 30 years due to an increasing population of immunocompromised patients who have cancer, AIDS or have received transplants. Antioxidants are substances that even at low concentration significantly delay or prevent oxidation of easily oxidizable substrates[4]. Antioxidants inhibit or delay oxidation which appears to have a role in the prevention of many diseases[5]. The applications of antioxidants are industrially widespread in order to prevent the oxidative degradation of polymers, auto-oxidation of fats, synthetic and natural pigments discoloration, etc. There is an increased interest of using antioxidants for medical purposes in the recent years[6-8].

Charge-transfer (CT) complexes were for a long time believed to have an important role in biological systems[9]. Protonic charge transfer complexes were first introduced by Matsunaga and coworkers[10]. Pauling regarded the hydrogen bond as a special case of charge transfer interaction[11]. Chloranilic acid (CA) and picric acid (PA) form salts

or charge transfer complexes with many organic compounds particularly with aromatic and aliphatic amines[12,13]. Stavudine is a synthetic thymidine nucleoside analog that is effective in the treatment of HIV[14-17]. The effect of stavudine on HIV reverse transcriptase are far more potent than its effects on cellular DNA polymerases and mitochondrial DNA synthesis, thus permitting its use therapeutically as part of highly active antiretroviral therapy(HAART) regimens[18,19].

In connection with such studies, the present paper reports the molecular complexes formed during the reaction of stavudine (**1**) and its intermediates such as 5-methyl-1-(2', 3',5'-tri-O-methanesulfonyl-β-D-ribofuranosyl)uracil (**2**) and 2-((phenoxy carbonyl) methyl)-tetrahydro-5-(5-methyl-4-oxopyrimidin-1(4H)-yl)furan-3-yl methanesulfonate (**3**) as an electron donor with chloranilic acid (CA) and picric acid (PA) as electron acceptors. These synthesized complexes were characterized by different spectral analyses and biological results were reported in this paper. On the basis of their activity, these complexes were identified as viable leads for further studies.

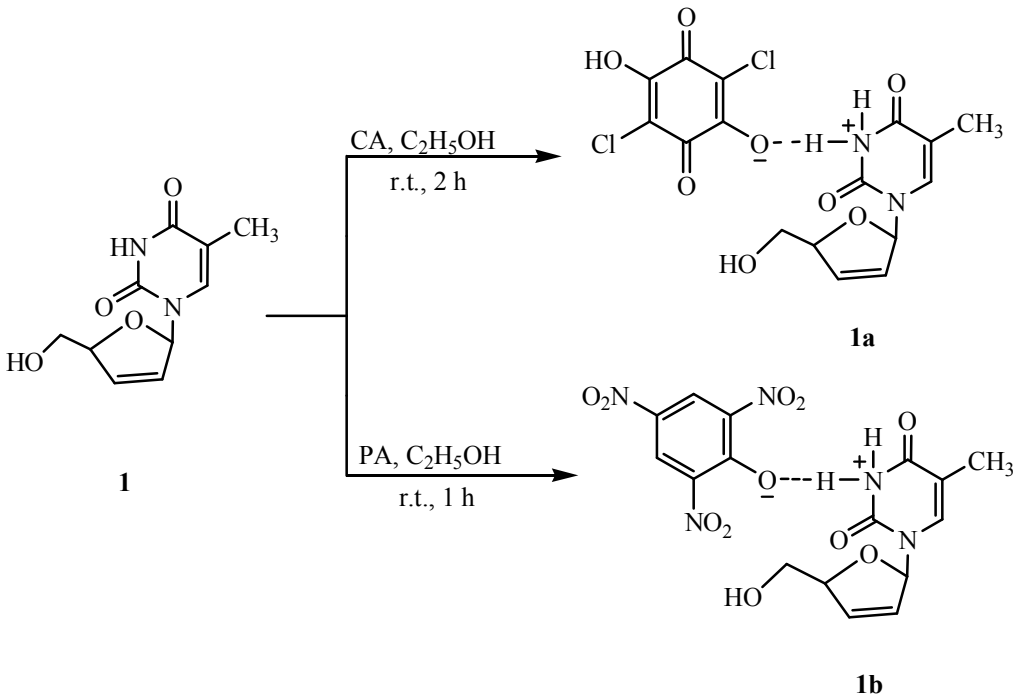
EXPERIMENTAL

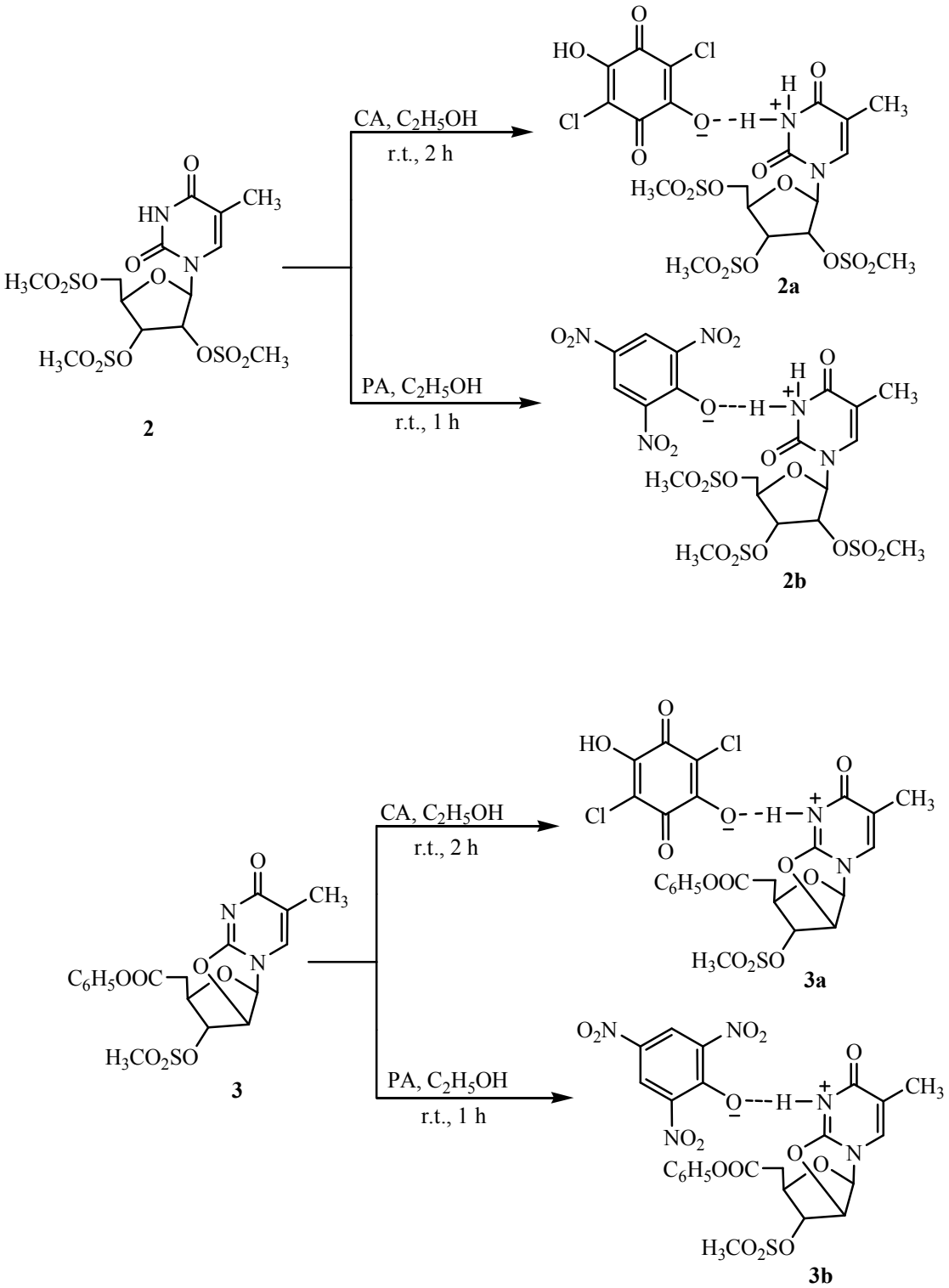
All solvents and reagents were purchased from Sigma-Aldrich, India. Melting points were determined by Veego Melting Point VMP III apparatus. Elemental analyses were recorded on VarioMICRO superuser V1.3.2 Elementar. The UV-visible spectra were recorded on Analytikjena Specord 50 UV–vis spectrophotometer with quartz cell of 1.0 cm path length in DMSO. The FT-IR spectra were recorded using KBr discs on FT-IR Jasco 4100 infrared spectrophotometer. ^{1}H NMR spectra were recorded on Bruker DRX -500 spectrometer at 400 MHz using d_6-DMSO as solvent and TMS as an internal standard. CT-complexes **1a**, **1b**, **2a, 2b**, **3a** and **3b** were synthesized by the method summarized in Scheme 1. The physical data of synthesized complexes are given in Table **1**.

Table 1 Physical data of synthesized complexes

Complex	Mol. Formula	Mol. Wt	M. R (^{0}C)	Yield (%)	λ_{max} (nm)
1a	$C_{16}H_{14}Cl_2N_2O_8$	433.20	248-251	61.4	525
1b	$C_{16}H_{15}N_5O_{11}$	453.32	188-190	68.2	430
2a	$C_{19}H_{22}Cl_2N_2O_{16}S_3$	701.48	228-230	64.6	510
2b	$C_{19}H_{23}N_5O_{19}S_3$	721.60	108-110	62.9	420
3a	$C_{24}H_{20}Cl_2N_2O_{12}S$	631.39	174-176	59.1	505
3b	$C_{24}H_{21}N_5O_{15}S$	651.51	113-115	61..2	415

Scheme 1





Synthesis of [(Stavudine) (CA)] (1a)

The complex, **1a** was synthesized by mixing Stavudine (0.9 g, 4 mmol) in ethanol (10 ml) with chloranilic acid (0.84 g, 4 mmol) in the same solvent. The mixture was stirred at room temperature for 2 h, where the solid precipitated after the reduction of the volume of the solvent. The separated precipitate was filtered off, washed several times with diethyl ether (2 × 0.5 ml) and dried in vacuum over $CaCl_2$. The product was purified by recrystallization using methanol solvent. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.53 (s, 1H), 7.55 (s, 1H), 6.45 (d, 1H), 5.76 (t, 2H), 5.10 (s, 2H), 4.58 (t, 1H), 4.51-4.42 (d, 2H), 2.97 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3431 (O-H), 3134 (N-H), 3045 (C-H), 1683 (C=O), 1462 (C=C), 1130 (C-O), 1064 (C-N), 681 (C-Cl). Anal. Calcd. for $C_{16}H_{14}Cl_2N_2O_8$ (in %): C-44.36, H-3.26, N-6.47. Found. C-44.65, H-3.07, N-6.24.

Synthesis of [(Stavudine)(PA)] (1b)

The complex, **1b** was synthesized by adding Stavudine (0.9 g, 4 mmol) in ethanol (10 ml) with picric acid (0.92 g, 4 mmol) in the same solvent. The mixture was stirred at room temperature for 1 h, where the solid precipitated after the reduction of volume of the solvent to the half. The precipitate was filtered off, washed several times with diethyl ether, and then dried over $CaCl_2$. The product was purified by recrystallization using methanol solvent^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.54 (s, 1H), 8.56 (s, 2H), 7.51 (s, 1H), 6.47 (d, 1H), 5.72 (t, 2H), 5.10 (s, 2H), 4.54 (t, 1H), 4.50-4.41 (d, 2H), 3.00 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3421 (O-H), 3153 (N-H), 3054 (C-H), 1642 (C=O), 1550 (NO_2), 1473 (C=C), 1131 (C-O), 1063 (C-N). Anal. Calcd. for $C_{16}H_{15}N_5O_{11}$ (in %): C-42.39, H-3.34, N-15.45. Found. C-42.35, H-3.12, N-15.17.

Synthesis of [(5-methyl-1-(2', 3',5'-tri-O-methane sulfonyl-β-D-ribofuranosyl)uracil) (CA)] (2a)

The complex, **2a** was synthesized according to the method described for the complex, **1a** employing **2** (2.0 g, 4 mmol) and CA (0.84 g, 4 mmol) to afford **2a**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.68 (s, 1H), 7.53 (s, 1H), 5.97 (s, 1H), 5.52 (s, 1H), 5.31 (s, 1H), 5.04 (s, 2H), 4.55 (t, 1H), 4.53-4.44 (d, 2H), 3.33-3.22 (s, 9H), 1.75 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3639 (O-H), 3235 (N-H), 3002 (C-H), 1685 (C=O), 1469 (C=C), 1354 (SO_2), 1132 (C-O), 1075 (C-N), 662 (C-Cl). Anal. Calcd. for $C_{19}H_{22}Cl_2N_2O_{16}S_3$ (in %): C-32.53, H-3.16, N-3.99. Found. C-32.23, H-3.22, N-4.07.

Synthesis of [(5-methyl-1-(2', 3',5'-tri-O-methane sulfonyl-β-D-ribofuranosyl)uracil)(PA)] (2b)

The complex, **2b** was synthesized according to the method described for the complex, **1b** employing **2** (2.0 g, 4 mmol) and PA (0.92 g, 4 mmol) to afford **2b**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.56 (s, 2H), 7.54 (s, 1H), 5.96 (s, 1H), 5.54 (s, 1H), 5.34 (s, 1H), 5.03 (s, 2H), 4.55 (t, 1H), 4.52-4.45 (d, 2H), 3.34-3.22 (s, 9H), 1.76 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3563 (O-H), 3186 (N-H), 3020 (C-H), 1633 (C=O), 1557 (NO_2), 1471 (C=C), 1360 (SO_2), 1132 (C-O), 1074 (C-N). Anal. Calcd. for $C_{19}H_{23}N_5O_{19}S_3$ (in %): C-31.62, H-3.21, N-9.71. Found. C-31.84, H-3.55, N-9.97.

Synthesis of [(2-((phenoxycarbonyl)methyl)-tetra hydro-5-(5-methyl-4-oxopyrimidin-1(4H)-yl)furan-3-yl methanesulfonate) (CA)] (3a)

The complex, **3a** was synthesized according to the method described for the complex, **1a** employing **3** (1.7 g, 4 mmol) and CA (0.84 g, 4 mmol) to afford **3a**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.64 (s, 1H), 7.21 (m, 5H), 5.97 (s, 1H), 5.51 (s, 1H), 5.30 (s, 1H), 5.12 (s, 1H), 4.52 (t, 1H), 4.55-4.41 (d, 2H), 3.33 (s, 3H), 2.35 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3302 (O-H), 3112 (N-H), 3012 (C-H), 1675 (C=O), 1463 (C=C), 1352 (SO_2), 1130 (C-O), 1072 (C-N), 742 (C-Cl). Anal. Calcd. for $C_{24}H_{20}Cl_2N_2O_{12}S$ (in %): C-45.65, H-3.19, N-4.44. Found. C-45.43, H-3.01, N-4.27.

Synthesis of [(2-((phenoxycarbonyl)methyl)-tetra hydro-5-(5-methyl-4-oxopyrimidin-1(4H)-yl)furan-3-yl methanesulfonate)(PA)] (3b)

The complex, **3b** was synthesized according to the method described for the complex, **1b** employing **3** (1.7 g, 4 mmol) and CA (0.84 g, 4 mmol) to afford **3b**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.56 (s, 2H), 7.62 (s, 1H), 7.19 (m, 5H), 5.95 (s, 1H), 5.50 (s, 1H), 5.31 (s, 1H), 5.11 (s, 1H), 4.53 (t, 1H), 4.53-4.41 (d, 2H), 3.31 (s, 3H), 2.34 (s, 3H). FT-IR (KBr, ν/cm^{-1}): 3423 (O-H), 3142 (N-H), 3024 (C-H), 1631 (C=O), 1558 (NO_2), 1470 (C=C), 1365 (SO_2), 1128 (C-O), 1064 (C-N). Anal. Calcd. for $C_{24}H_{21}N_5O_{15}S$ (in %): C-44.24, H-3.25, N-10.75. Found. C-44.13, H-3.01, N-10.27.

BIOLOGICAL EVALUATION

***In Vitro* Evaluation of Antibacterial Assay**

Antibacterial activity of the synthesized compounds was determined against gram-positive bacteria (*Bacillus subtillis*, *Salmonella typhi*) and gram-negative bacteria (*Xanthomonas malvacearum* and *Escherichia coli*) in DMF by disc diffusion method on nutrient agar medium[20]. The sterile medium (Nutrient Agar medium, 15 ml) in each petriplates was uniformly smeared with cultures of gram +ve and gram –ve bacteria. Sterile discs of 10 mm diameter (Hi media) were made in each of the petriplates, to which 50 µL of the different synthesized compounds were added. The treatments also included 50 µL of DMF and streptomycin as negative and positive control for comparison. Each compound was assessed in triplicate. The plates were incubated overnight at 25 ± 2 ºC and then the inhibition zones were measured in millimeters. The results of the antimicrobial activity for the synthesized complexes were recorded in Table **2**.

***In Vitro* Evaluation of Antifungal Assay**

The synthesized complexes were screened for their antifungal activity against *Fusarium oxysporum* in DMF by poisoned food technique[21]. Potato Dextrose Agar (PDA) media was prepared and about 15 ml of PDA was poured into each petri plate and allowed to solidify. 5 mm disc of seven days old culture of the test fungi was placed at the center of the petri plates and incubated at 26 °C for 7 days. After incubation the percentage inhibition was measured and three replicates were maintained for each treatment. Activity of each compound was compared with standard drug nystatin. All the synthesized complexes were tested (at

the dosage of 500 µl of the complexes /petriplate, where concentration was 0.1 mg/ml) by poisoned food technique.

DPPH Radical Scavenging Assay

The free radical scavenging activity of the synthesized complexes was studied *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay method[22]. Stock solution of the drug was diluted to different concentrations in the range of 100-200 µg/ml in methanol. Methanolic solution of the complexes (2 ml) was added to 0.003% (w/v) methanol solution of DPPH (1 ml). The mixture was shaken vigorously allowed to stand for 30 min, absorbance at 517 nm was determined and the percentage of scavenging activity was calculated. Ascorbic acid was used as the reference compound. All tests and analyses were done in duplicate and the results were averaged. Results are presented in Table **3**. The inhibition ratio (I %) of the tested compounds was calculated according to the following equation:

$$I\ \% = (Ac\text{-}As) / Ac \times 100$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

RESULTS AND DISCUSSION

Elemental Analyses and UV-Visible Spectra

Reaction of electron donors with electron acceptors resulted in the formation of stable charge-transfer complexes with a donor–acceptor ratio of 1:1, and was formulated as (**1a**), (**1b**), (**2a**), (**2b**), (**3a**) and (**3b**) respectively. The elemental analyses data showed good agreement between the experimentally determined values and the theoretically calculated values within the limits of permissible error. These charge transfer complexes are stable in air, soluble in DMSO and DMF. The elemental analyses data confirm the stoichiometry and hence the molecular formula of the synthesized complexes. New bands were detected in the UV-visible spectra of the CT complexes. These bands are not exhibited by either donor or acceptors alone. The appearance of longer wavelength absorption band in the visible region in UV-visible spectrum owing to the charge transfer transition confirms the formation of molecular complexes.

FT-IR and ^{1}H NMR Spectra

The Infrared spectra of the molecular complexes of CA and PA with donors indicate that ν(C–Cl) of CA and $\nu(NO_2)$ of PA are shifted to lower wavenumber values upon complexation. The stretching frequency of C=O bond of the acceptor displays a shift to a higher wavenumber values upon complexation. Infrared spectra of the synthesized complexes show a strong band, indicating $\overset{+}{N}$–H···O$^-$ stretching vibration of the intermolecular hydrogen bond. The protonation of the NH group of the donor through one proton transfer from one of the acidic centre on the CA from one side (OH) of the basic centre. On the other hand, the intermolecular hydrogen bond occurs in the PA from the OH group to the basic central nitrogen atom of the donor. New signals are observed in ^{1}H NMR for the synthesized complexes assigned to $\overset{+}{N}$–H proton which resulted from the protonation of N atom of donors. The O–H signals of the free CA and PA were disappeared on complex formation. These data is agreed quite well with the elemental analyses, UV-visible and FT-IR studies.

Antibacterial Activity

The investigation of antibacterial screening data revealed that stavudine and its intermediates with synthesized complexes were evaluated and compared with standard drug, streptomycin. The stavudine and its complex, **1a** and **1b** showed significant active inhibitory against *B. subtillu* (zone of inhibition 19-22 mm) in the order of **1a** > **1b** > **1** compared to standard drug. Stavudine intermediates and its complexes, **2a**, **2b**, **3a** and **3b** showed weak active inhibitory against *B. subtillu* (zone of inhibition < 7 mm). Stavudine and its intermediates with synthesized complexes showed weak activity with the zone of inhibition in the range of < 7 mm against *E. colis* compared with standard drug.

Antifungal Activity

The antifungal activity of the stavudine, its intermediates and synthesized complexes were evaluated and compared with standard drug nistatin. Complexes, **1a** showed significant antifungal activity with the inhibition 81 % against *F. oxysporum* compared with **1**, **2**, **3**, **1b**, **2a**, **2b**, **3a** and **3b**, respectively. Among the synthesized complexes inhibitory activity in the order **1a** > **1b** > **2a** > **2b** > **3a** > **3b** against tested fungi. From the results obtained, it reveals that the significant inhibitory activity is probably due to the presence of hydroxyl group in **1a**. The CT-complexes of donors with chloranilic acid showed more antimicrobial activity compared with salts of picric acid. Antimicrobial screening results of the tested complexes are shown in Table **2**.

Antioxidant Activity

Absorbance of the stable radical DPPH$^\bullet$ was measured at 517 nm for different concentrations of newly synthesized complexes. Antioxidant activity results of the tested compounds are shown in Table **3**. Complex, **2a** showed moderate antioxidant activity (48.5 %) which was comparable to that of the standard ascorbic acid (93.4 %) at 200 µg/ml (Figure **1**). Antioxidant activity of stavudine and its intermediates with synthesized complexes showed the following order: **2a** > **2b** > **2** > **1a** > **3a** > **1b** > **3b** > **1** > **3**. From the results

obtained, it reveals that the moderate inhibitory activity is probably due to the presence of methanesulfonate group in **2a**. Further, CT-complexes of donors with chloranilic acid are more antioxidant activity compared with picric acid salts.

Table 2 *In vitro* antibacterial and antimicrobial activities of compounds

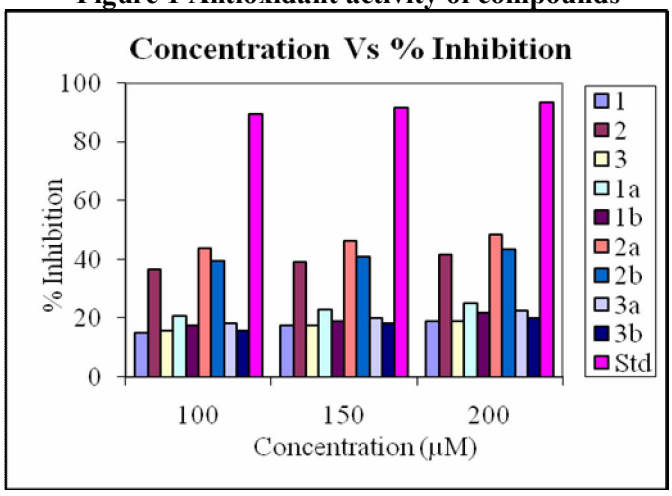
Compound	Zone of inhibition in mm				% Inhibition
	Gram-positive bacteria		Gram-negative bacteria		
	B. subtillu	***S. typhi***	***X. malvacearum***	***E. colis***	***F. oxysporum***
1	17	21	21	-	74
2	-	21	20	-	58
3	-	20	20	-	53
1a	19	22	22	-	81
1b	18	21	21	-	76
2a	-	21	21	-	64
2b	-	20	19	-	62
3a	-	21	21	-	58
3b	-	20	19	-	55
Streptomycin	21	24	23	20	-
Nystatin	-	-	-	-	90

Zone of inhibition: - = < 7 mm

Table 3 Results of DPPH radical scavenging assay

compound	% Scavenging (Mean ± SEM) of duplicates		
	100 µg/ml	**150 µg/ml**	**200 µg/ml**
1	15.1 ± 0.002	17.5 ± 0.001	19.4 ± 0.001
2	36.8 ± 0.001	39.2 ± 0.002	41.8 ± 0.001
3	15.8 ± 0.001	17.5 ± 0.001	19.3 ± 0.001
1a	20.8 ± 0.002	23.1 ± 0.001	25.3 ± 0.003
1b	17.5 ± 0.001	19.2 ± 0.002	21.8 ± 0.001
2a	43.8 ± 0.001	46.3 ± 0.001	48.5 ± 0.001
2b	39.7 ± 0.003	41.1 ± 0.001	43.7 ± 0.003
3a	18.4 ± 0.002	20.1 ± 0.003	22.8 ± 0.001
3b	15.9 ± 0.001	18.4 ± 0.002	20.3 ± 0.002
Ascorbic acid	89.4 ± 0.001	91.7 ± 0.002	93.4 ± 0.001

Figure 1 Antioxidant activity of compounds



CONCLUSION

In conclusion, complexes of stavudine and its intermediates with chloranilic and picric acids were synthesized in good yield and their antimicrobial and antioxidant activities have been evaluated. The synthesized complexes were confirmed by elemental analyses, UV-visible, FT-IR and ^{1}H NMR spectral studies. Complex, **1a** demonstrated significant inhibition against all the strains tested. The antioxidant activity revealed that complex, **2a** is moderate antioxidant activity compared with standard drug.

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