Metal II complexes of ethambutol as good enzyme inhibitor and promising antioxidant

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Abstract: Ethambutoldihydrogenchloride (EMB) with chemical formula $C_{10}H_{24}N_2O_2.2HCl$ is ethane-1,2-diamine in which one hydrogen attached to each of the nitrogen is substituted by a 1-hydroxybutan-2-yl group (*S,S*-configuration). It is an FDA approved drug and has been used for treatment of tuberculosis since 1960's. Prolong use of EMB has a side effect of visual impairment and in literature it is related with the depletion of Zn metal from the body. As it is a good chelating agent, many metal II complexes have been synthesized with anti-tubercular activity. The purpose of this work was to synthesize metal II complexes of EMB and to evaluate their antioxidant activity along with enzyme inhibition activity (acetylcholine esterase and protease). The metals used for complex formation were Co, Zn, Fe, Cu and Ni. IR spectral data and physical parameters supported the complex formation. The obtained results showed the synthesized complexes as notable antioxidants and enzyme inhibitors.

Keywords: Ethambutoldihydrogenchloride (EMB), metal II complexes, antioxidant activity, acetylcholine esterase inhibition, protease inhibition.

INTRODUCTION

Ethambutoldihydrogenchloride (EMB) is a dextro-2,2′-(ethylene-diimino)-di-1-butol dihydrogenchloride. It is adiamine molecule and synthesized by the reaction of 1,2-dihaloethane with chirally pure (*S*)-2-amino-1-butanol. First time, Wilkinson and coworkers reported this molecule for its anti-tubercular activity from Lederle laboratories (Wilkinson *et al.*, 1961; Thomas *et al.*, 1961). In combination with other drugs, it has been used for the treatment of tuberculosis. Tuberculosis has emerged as the biggest health problem in the world since 1980's. Almost two million people have been reported to be affected by this disease mainly in the developing countries (Kamzui *et al.*, 1980;de Oliveira Neves *et al.,* 2012). Originally EMB was used in the form of racemic mixture but later on only *D*-form. It was found that *D*-form is responsible for the treatment and *L*-form was found to be toxic (Su-Ann, 2006). Its mechanism of action includes inhibition of growth of mycobacterium by targeting arabinosyltransferases, required for the biosynthesis of arabinogalactan; A component of mycobacteria cell wall (Lee *et al.*, 1995; Zhang *et al.*, 2007). Furthermore, it has been investigated that the course of action of EMB in physiological systems involves formation of chelate complexes with bivalent cations (Maddry *et al.*, 1996). The causative agent of tuberculosisis *Mycobacterium tuberculosis* which grows at very slow rate of 16 to 20 hours (Su-Ann, 2006). Thus medication is required for a long duration, that is, 6 to 9 months (Yukitaka *et al.*, 2004). The side effects of prolonged use of EMB may result in restriction of visual field and loss of color discrimination. These side effects have been proposed to be related to the depletion of copper and zinc in human system (Lei bold and Ann, 1956).

The EMB has been used to form complexes with metals including Copper, Nickel, Zinc and Platinum (Lee and Benet, 1978; Weismann, 1986; Benedetti *et al.*, 2002). Ni and Cu complexes of EMB have been used to evaluate their anti-tubular activity (Mathrusri *et al.*, 2006). No other activity has been performed by these complexes so far. This study is about the synthesis of Metal II complexes of EMB with different metals like Co, Zn, Fe, Ni and Cu; and to evaluate their *in vitro* antioxidant and enzyme inhibition activities. The enzyme inhibition activity included acetylcholine esterase enzyme (AChE) inhibition and protease (trypsin) inhibition.

Antioxidants are such species that stops the free radical attack and avert them ageing and different diseases that are related with the oxidative damage inside the body (Rice-Evans *et al.*, 1996). The diseases that can be prevented by antioxidants include cancer, emphysema, cirrhosis, atherosclerosis, arthritis and all other degenerative diseases (Halliwell and Gutteridge, 1984).

The acetylcholine esterase enzyme (AChE) hydrolyzes the acetylcholine into choline and acetate group and prevents the neurological damaging effect of acetylcholine (Luis *et al.*, 2006). Neurological disorders such as Alzheimer's disease, myasthenia gravis, ataxia and senile dementia are prevented by the inhibitors of AChE (Ahmad *et al.*, 2003). Protease enzyme plays an important role in the normal physiological function of the body. But over activity of this enzyme can result in the diseases like cancer, pulmonary emphysema, arthritis (Giovanni and David, 2005).

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MATERIALS AND METHODS

Chemicals

Ethambutoldihydrogenchloride (EMB) was obtained from industry. $FeSO_4.7H_2O$ was obtained from Riedel-de Haen; $CoCl_2.6H_2O$, $ZnCl_2$, $CuSO_4.5H_2O$, $NiCl_2$, $FeCl_3$ andammonium molybdate were obtained from Merck (Germany). FC reagent (Folin-Ciocalteu's reagent), DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), BHT (Butylatedhydroxytoluene), ATCI (Acetylthiocholine iodide), DTNB (5,5-dithiobis-2-nitrobenzoic acid)and PMSF (Phenylmethanesulfonyl fluoride) were obtained from Sigma Chemical Company Ltd. (USA). DMSOtrypsin from bovine pancreas and Na-benzoyl-DL-arginine-paranitroanilide- HCl (BApNA) was purchased from Fluka. All the chemicals were purchased through local suppliers. The erythrocytes (acetylcholine esterase) obtained from the Biochemistry Laboratory, Mayo Hospital, Lahore, Pakistan.

General method for synthesis of metal II complexes

Complexes of different metals with EMB were synthesized by reacting particular metal salt and EMB in a molar ratio of 1:2. By using water as reaction media it was refluxed for 150min (2 hrs and 30min). Crystals of metal II complexes appeared on standing at room temperature. The formed complexes were dried and subjected to further analysis.

Antioxidant activity methods

Following antioxidant assays were used for the determination of antioxidant activity of the metal II complexes of EMB.

Sample preparation for antioxidant activity

Samples were prepared by weighing 0.02g of each of the complex and dissolving it in 20mL of methanol.

Total antioxidant activity determination

Total antioxidant activity was determined byphosphomolybdenum complex formation method (Prieto *et al.*, 1999). First of all a reagent was made with the composition 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. Then 500µg/mL of each of complex was taken with the addition of 4mL of prepared reagent. The test tubes were kept in water bath at 95°C for 90min. The sample test tubes were cooled to room temperature and absorbance was recorded at 695 nm. The antioxidant activity was determined against BHT. Results were calculated as mean of three independent experiments.

DPPH radical scavenging activity

DPPH radical scavenging activity of the samples was determined by comparing with standard antioxidant activity of BHT (Lee and Shibamoto, 2001). Different concentrations of sample such as 1000µg/mL, 500µg/mL, 250µg/mL were made in test tubes. To these concentrations 3.0mL of 0.1m Mmethanolic solution of DPPH was added. Test tubes were shaken and kept at room temperature for 60 min. Blank absorbance of methanol was taken at 517nm with help of spectrophotometer. Higher the free radical scavenging activity lower was the absorbance. The percentage of de-coloration of DPPH was calculated by using formula:

Antiradical activity = $\{(R_{control}- R_{sample}) / R_{control}\} \times 100$

This process was repeated three time and mean values were calculated as final results.

Ferric reducing antioxidant power (FRAP)

FRAP assay method reported by Benzie and Strain was applied after some modifications (Benzie *et al.*, 1996). Fresh solution of FRAP was prepared by mixing 2.5mL TPTZ with 2.5mL ferric chloride hexa hydrate ($FeCl_3.6H_2O$) solution. Then 25mL acetate buffer (pH 3.6) was added and warmed at 37°C.150µL of FRAP solution was added to 50µL of each sample solution and kept in dark for 30 min. After 30min, a colored product of ferrous tripyridyltriazine complex was formed and absorbance was measured at 593nm. The FRAP values were expressed as mM of trolox equivalents (TE) per mL of sample by using standard calibration curve constructed for different concentrations of trolox.

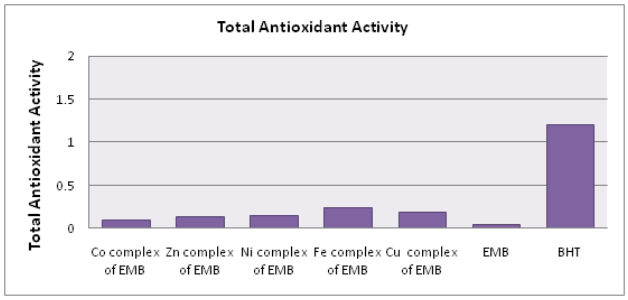

Fig. 1: Graphical representation of results of total antioxidant activity.

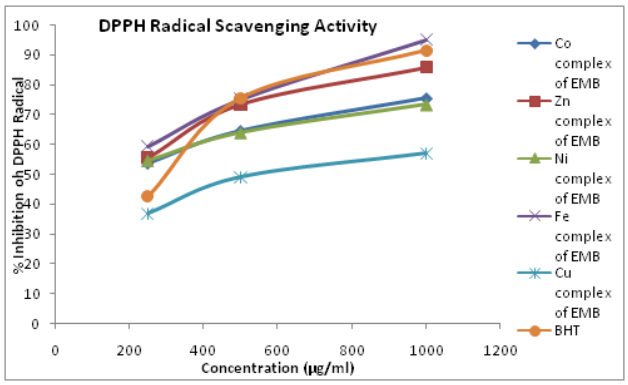

Fig. 2: Graphical representation of results of DPPH radical scavenging activity.

Total phenolic content determination (TPC)

A reported spectroscopic method was used for determination of total phenolic contents (Makkar *et al.*, 1993). To every 0.1mL (0.5mg/mL) of each sample,

2.8mL of sodium carbonate (10%) and 0.1mL Folin-Ciocalteu (FC) reagent/mL was added. The samples were kept for 40 min and then absorbance was determined at 725nm. Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract by using the standard curve obtained at different concentrations of gallic acid. Results were expressed in GAE mg/g.

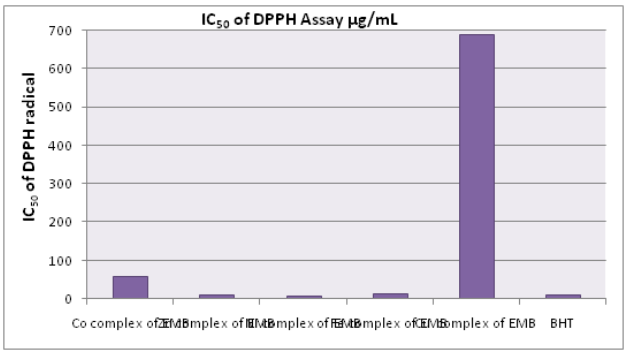

Fig. 3: Graphical representation of results of IC_{50} of DPPH activity.

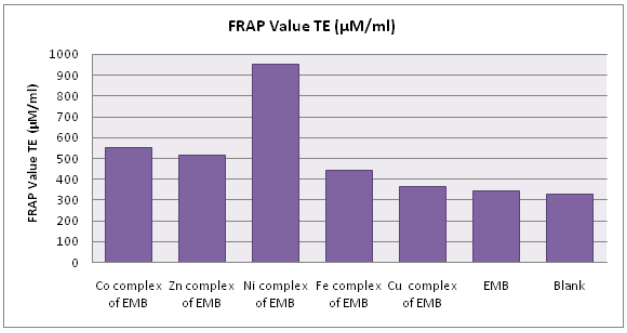

Fig. 4: Graphical representation of results of FRAP value.

Enzyme inhibition assays

Two *invitro*enzyme inhibition methods were applied.

Acetylcholine esterase (AChE) inhibition assay

AChE inhibition was assayed by the reported method of Ellman after small modifications (Ellman *et al.*, 1961; Shah war *et al.*, 2010). A mixture containing 2.8mL of 0.1M phosphate buffer (pH 7.8), 100µL of acetylchline esterase (erythrocytes), 200µL of each fraction solution at different concentrations (150, 100, 50 and 30mg/mL) and 100µL of DTNB was prepared. This mixture was kept for 15min at 37°C in an incubator. After 15 min, 200µL substrate (acetylthiocholine) was added for reaction to initiate. After keeping it for 30min, the hydrolysis of acetylthiocholine was measured by taking the absorbance at 412nm. For positive control, Galanthamine was used and the negative control was established without adding substrate. The method was repeated three time and mean values were calculated. Percentage inhibition was calculated with help of following formula:

$$\% \text{ inhibition} = [(E_A - E_B)/ E_A] \times 100$$

In this equation; E_A is the activity of the enzyme without sample and E_B is the activity of enzyme with test sample.

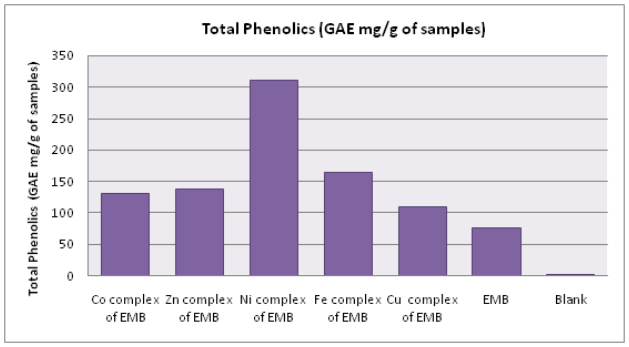

Fig. 5: Graphical representation of results of total phenolics.

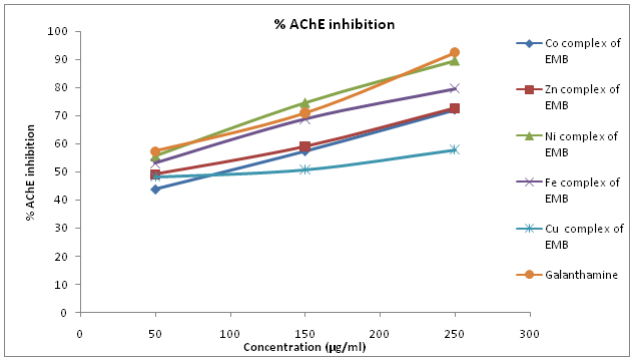

Fig. 6: Graphical representation of results of % inhibition of AchE.

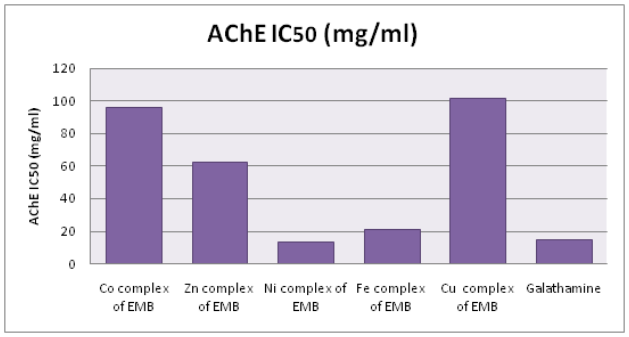

Fig. 7: Graphical representation of results of IC_{50} values for AChE inhibition.

Protease inhibition assay

Protease inhibition assay is reported by aspectroscopic method (Shahwar *et al.*, 2011). Trisbuffer (100mM) of pH 7.5 was prepared by dissolving 12.1g of tris (hydroxymethyl) amino methane in distilled water along with HCl (5M) to maintain pH of 7.5. For preparing stock solution of trypsin 2mg of trypsin were dissolved in 10mL HCl (1.0mM). DMSO was used to dissolve BapNA (20mg/mL). Mixture containing 100µL inhibitor (2 mg/mL) and enzyme (0.3mL) was kept in incubator at 37°C for a time period of 15min.After this 0.6mMsubstratewas added and the final volume was

adjusted to 2.5mLusing tris buffer. This mixture was kept in incubator at 37°C for 30min. For quenching purpose, 30% acetic acid was added and absorption was taken at 410nm. The positive inhibitor used was PMSF. Along with positive inhibitor, blank sample and negative inhibitor (no enzyme) were also prepared by adding solvent and inhibitor respectively. The following formula was used for the calculation of the percentage inhibition:

% Inhibition = $[(A_{Blank} – A_{Test})/ A_{Blank}] \times 100$

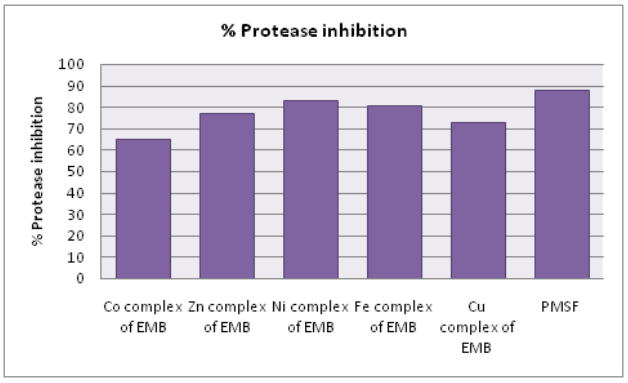

Fig. 8: Graphical representation of results of % inhibition of Protease.

STATISTICAL ANALYSIS

All the readings were taken three times and Statistical software was used to perform statistical analysis. All the results are expressed as mean ± S.E.M. In order to determine statistical analysis one way analysis of variance (ANNOVA) was used followed by post-hoc Tukey's test.

RESULTS

Synthesis of complex

For the synthesis of metal II complexes of EMB, distilled water was used as reaction medium. The reported method of complex formation included methanol as reaction medium (Mathrusri *et al.*, 2006) but impurity of unreacted EMB has been noted in this medium as shown by melting points. When distilled water was used as reaction medium, sharp melting points were obtained which were all lower than EMB melting point. The physical properties like melting point, colour, solubilities and IR data are cited in table 1.

IR studies

By comparing the IR spectra of EMB with that of the complexes, we can interpret that main peaks of the ligand at 3320cm^{-1}, 1055cm^{-1} and 1550cm^{-1}were retained in the complexes (Mathrusri *et al.*, 2006). An extra peak appeared in the spectra of complexes at 1700-1600cm^{-1}indicating the formation of complexes. The peak between 1700-1600cm^{-1} was very sharp for each complex expect Cu complexes of EMB. Another difference that was noted in comparison of IR spectra of EMB and complexes, peaks were very sharp in EMB spectra indicating no inter molecular hydrogen bonding but the spectra of complexes showed broad peak indicating inter molecular hydrogen bonding. It can also be predicted that during complex formation the -OH group did not contribute but its –NH group which is involved in complex formation. This trend of peak broadening is more in EMB complex of Co and lowest in EMB complex of Cu. The frequencies of prominent peaks are cited in table 1.

Antioxidant study

The results of antioxidant activity of the complexes are summarized in tables 2 and 3.

Total antioxidant activity

For determination of total antioxidant activity spectroscopic ally, phosphomolybdenum method was used. Comparison of the samples was performed with the reference standard antioxidant (BHT). Fe complex of EMB showed the highest antioxidant activity 0.244±0.87followed by EMB complexes of Cu, Ni, Zn and Co. EMB itself showed very low values of total antioxidant activity. The results are presented graphically in fig. 1.

DPPH radical scavenging activity

The results of percentage inhibition of DPPH radical and values of $IC_{50}$are shown in the fig. 2 and fig. 3.The results showed that for each of the sample the absorption decreased with decrease in concentration. Fe complex of EMB showed the highest value of absorption, 95±0.51,at concentration 1000µg/mL followed by the Zn, Co and Cu. IC_{50}value were calculated for each of the sample by plotting curves. Lower the value of IC_{50}, better the DPPH scavenging activity of the complex. Ni complex of EMB showed lowest IC_{50}value 7.5±0.12 followed by Zn, Fe, Co and Cu with lowest activity among the complexes. IC_{50}value of EMB was very large indicating that it showed no DPPH scavenging activity. So it was included for the graphical representation.

Ferric reducing antioxidant power (FRAP)

FRAP test was performed to determine the reducing potential of the samples. The results are shown in fig. 4.The results of this test depicted that Ni complex of EMB showed highest FRAP value (948±0.018) followed by Co complex of EMB showing value of (551±0.70) > Zn complex of EMB (513±0.40) > Fe complex of EMB (441±0.021) > Cu complex of EMB (364±0.14).

Total phenolic content determination (TPC)

The results are shown graphically in fig. 5.Highest value was shown by Ni complexes of EMB (310±0.067) followed by Fe complex of EMB (163±0.15)> Zn complex of EMB (137±0.86)> Co complex of EMB (130±1.02)> Cu complex of EMB (109±0.27). EMB showed the lowest value.

Table 1: Physical properties of metal II complexes of EMB

Compound	Color	Melting point (°C)	Solubility	IR Frequency (cm^{-1})
Co(EMB)	Violet	88	Soluble in H_2O/CH_3OH but insoluble in $CHCl_3$/EtOAc	600(s), 1050(s), 1480(m), 1550(m)1650 (s), 2800(def), 2980(w), 3320(s, br)
Fe(EMB)	Orange red	94	Soluble in H_2O/CH_3OH but insoluble in $CHCl_3$/EtOAc	600(s) 1050(s),1480(s)1550(s), 1650(m, br) 2800(def) 2980(m),3320(s, br)
Cu(EMB)	Green	66	Soluble in H_2O/CH_3OH but insoluble in $CHCl_3$/EtOAc	600(s), 1050(s), 1480(s), 1550(s), 1650(w), 2800(s), 2980(m), 3320(s)
Zn(EMB)	White	120	Soluble in H_2O/CH_3OH but insoluble in $CHCl_3$/EtOAc	600(s) 1050(s) 1480(s) 1550(s),1650(m, br), 2800(s, br), 2980(s), 3320(s,br)
Ni(EMB)	Green	128	Soluble in H_2O/CH_3OH but insoluble in $CHCl_3$/EtOAc	600(s) 1050(s) 1480(s) 1550(s),1650(m, br), 2800(m, br), 2980(m), 3320(s, br)
EMB	White powder	207	Soluble in H_2O/CH_3OH but partially soluble in $CHCl_3$	600(s), 1050(s), 1480(s), 1550(s), 2800(s), 2980(s), 3320(s)

Note: s = strong; w = weak; m = medium; def = deformed; br = broad

Table 2: Free radical scavenging activity of the metal II complexes of EMB using DPPH

Compound	Concentration in assay (µg/mL)	% Scavenging of DPPH ± S.E.M [a)]
Co(EMB)	1000	75.55±0.09
	500	64.58±0.32
	250	53.78±0.03
Zn(EMB)	1000	85.67±0.05
	500	73.45±0.17
	250	55.67±0.02
Ni(EMB)	1000	73.45±0.40
	500	63.98±0.03
	250	54.56±0.076
Fe(EMB)	1000	95±0.51
	500	75.06±0.18
	250	59.34±0.43
Cu(EMB)	1000	57±0.056
	500	49±0.081
	250	36.78±0.31
EMB	1000	-75±0.01
	500	-66±0.043
	250	-31±0.10
BHT [b)]	1000	91.49±0.13
	500	75.54±0.07
	250	42.62±0.04

Table 3: IC_{50} values of DPPH activity, total antioxidant activity, FRAP values and total phenolics values of metal II complexes of EMB.

Compound	IC_{50} of DPPH Assay (µg/ml)±S.E.M [a)]	Total antioxidant activity ±S.E.M [a)]	FRAP Value TE (µM/mL)±S.E.M [a)]	Total Phenolics (GAE mg/g of extract) ±S.E.M [a)]
Co(EMB)	61±0.72	0.104±0.90	551±0.70	130±1.02
Zn(EMB)	11.98±0.67	0.136±0.02	513±0.40	137±0.86
Ni(EMB)	7.5±0.12	0.159±0.21	948±0.018	310±0.067
Fe(EMB)	13.69±0.06	0.244±0.87	441±0.021	163±0.15
Cu(EMB)	688.8±0.56	0.193±0.09	364±0.14	109±0.27
EMB	2375±0.32	0.045±0.035	339±0.92	76±0.39
BHT [b)]	12.10±0.29	1.2186±0.015		
Blank			326±0.54	1.15±0.051

Note: a) Standard error mean; b) Standard reference.

Table 4: AChE inhibition activity

Compound	Concentration in assay (µg/mL)	% Inhibition ± S.E.M[a)]	IC_{50} of ACTH Assay (µg/mL) ±S.E.M[a)]
Co(EMB)	250 150 50	72.09±0.01 57.33±0.14 43.77±0.21	95.81±0.02
Zn(EMB)	250 150 50	72.67±0.23 59.06±0.17 49.25±0.05	61.88±0.17
Ni(EMB)	250 150 50	89.57±0.43 74.59±0.32 55.63±0.12	12.84±0.32
Fe(EMB)	250 150 50	79.63±0.63 68.82±0.24 53.02±0.42	21.12±0.12
Cu(EMB)	250 150 50	57.95±0.13 50.88±0.02 48.29±0.11	101.66±0.87
Galanthamine[b)]	250 150 50	92.47±0.09 70.99±0.71 57.45±0.01	14.89±0.34

Note: a) Standard error mean; b) Standard reference.

Enzyme inhibition

The results for enzyme inhibition activity against AChE and protease enzymes are listed in table 4 and table 5.

AChE inhibition activity

The values of % inhibition by complexes at various concentrations andIC_{50}values are given in table 4 and graphical representations are shown in fig. 6 and fig. 7. A lower value of IC_{50} indicated more inhibition. Ni complexes showed lowest IC_{50} (12.84±0.32) followed by Fe complex 21.12±0.12> Zn complex of EMB (61.88±0.17) >Co complex of EMB 95.81±0.02 > Cu complex of EMB 101.66±0.87.

Table 5: Protease% inhibition

Compound	% Protease Inhibition ±S.E.M [a)]
Co(EMB)	64.58±0.12
Zn(EMB)	77.01±0.09
Ni(EMB)	82.89±0.34
Fe(EMB)	80.10±0.14
Cu(EMB)	72.78±0.01
PMSF[b)]	87.4±1.2

Note: a) Standard error mean; b) Standard reference

Protease inhibition

Results for percentage of protease inhibition are presented in the table 5 and graphical presentation is shown in fig. 8. Results indicated that Ni complexes of EMB showed the highest value of protease inhibition as82.89±0.34 followed by Fe complex of EMB 80.10±0.14 > Zn complex of EMB 77.01±0.09 > Cu complex of EMB 72.78±0.01 > Co complex of EMB 64.58±0.12.

DISCUSSION

The EMB metal complexes of Ni, Cu, Zn, Fe and Co showed good antioxidant activity as well as enzyme inhibition for AChE and protease enzyme. Total antioxidant activity determination by phosphomolybdenum is based on formation of green phosphate Mo (V) complex after reduction of Mo(VI) to Mo(V) by the sample analyte at acidic pH. Depending on the analyte structure, the transfer of electrons takes place (Miladi and Damak, 2008). DPPH scavenging was used to evaluate the antioxidant power due to the hydrogen donating ability or the radical scavenging. Chemistry of this method is such that when a substance having ability to donate hydrogen atom or transfer electrons is mixed with DPPH, it neutralizes the free radical character. The violet color is lost with the formation of reduced non radical form as 1,1-diphenyl-2-picryl hydrazine and is changed into yellow. With the increase in the percentage of radical inhibition, radical scavenging activity also increases (Huang *et al*., 2005). FRAP assay method takes into account the oxidation-reduction potential of samples. This method is based on the ability of the antioxidants to reduce ferric tripyridyltriazine (Fe(III)-TPTZ) complex to intense blue color ferrous tripyridyltriazine (Fe(II)-TPTZ) complex (Benzie *et al.*, 1996).The development of the color indicated that reductant (antioxidant) is present in the reaction mixture. Total phenolic contents are determined though FC (Folin-Ciocalteu) reagent. FC reagent is a yellow acidic solution composed of complex polymeric ions. These complex polymeric ions are formed from phosphomolybdic and phosphotungsticheteropoly acids. Phenolates are oxidized by this reagent which results in formation of blue colored complex

molybdenum- tungsten blue and this can be detected with help of a spectrophotometer at 725nm. It is calculated as gallic acid equivalents. The values of the absorbance at this wavelength were inserted in the equation y (absorbance) =0.006x+0.139. And in this way total amount of the phenolic contents were calculated.

The method for inhibition of Acetylcholine esterase (AChE) is based on the formation of a yellow colored anion of thionitrobenzoic acid by the hydrolysis of the DTNB. This is measured by taking absorbance at 412nm. More will be the absorption, good will be the inhibition. Results showed Ni complexes of EMB are good inhibitors of AChE and hence good for treatment of Alzheimer's disease. Protease inhibition was determined by spectroscopic method and here again Ni complexes of EMB remained prominent protease inhibitor.

CONCLUSION

Metal II complexes have been synthesized from EMB in an aqueous medium. This study showed metal ᵡᵡ complexes of EMB as good antioxidants and good inhibitors of two enzymes, that is, acetylcholine esterase and protease (trypsin). Among all the synthesized complexes, Ni complex of EMB showed good results of three methods of antioxidant activity evaluation out of four used i.e. DPPH radical scavenging, FRAP and TPC. Fe complex of EMB showed good results for total antioxidant method. Good antioxidant potential of these complexes showed their importance for the protection of body against some chronic diseases like cancer, cardio and cerebrovascular systems. The good enzyme inhibition results showed that they may be good resistant against the Alzheimer's disease (AD), pulmonary emphysema and arthritis. This study has demonstrated the importance of these metal II complexes of EMB in the field of medicine. These complexes can-not only be used for treatment of various diseases as well as we can predict the behavior of these complexes inside the body. Therefore rather using EMB as a salt, which is pro-complexing agent and has potential side effects, it can be used as metal complexes.

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