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Antitumor, antioxidant and antibacterial evaluation of new ligand of cinnemaldehyde and its Fe (III) and Mn (II) complexes

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Abstract

Cinnamomum zeylanicum is medicinal plant posse's significant biological activities. In this study, we investigated biological properties of the ligand and metal complexes synthesized from herb of *C.zeylanicum* (cinnemaldehyde) by chemical reactions. N^4 [(E, 2E)-3-Phenyl-2-propenylidene] isonicotinohydrazide abbreviated as NI were synthesized as ligand. Fe (III) and Mn (II) metal complexes of this ligand prepared. Characterization of the ligand and its complexes were made by microanalyses, ^{1}H NMR, ^{13}C NMR, FT-IR and UV–Visible spectroscopy. Cytotoxic, antioxidant and antibacterial activities of these compounds have been evaluated by MTT, DPPH, agar disc diffusion and agar dilution assays, respectively. These new compounds showed high Cytotoxic effect against K562, Jurkat and $T_{47}D$ cell lines. Our results showed that the metal complexes and their ligand, had weak antioxidant potential with RC_{50} value of 0.394, 0.724 and 1.429 mg/mL, respectively. The Fe (III) complex had the highest cytotoxic effects on K562, Jurkat and $T_{47}D$ with IC_{50} value of 86.12, 77.86, 118.52, respectively. The Fe (III) complex had the highest antibacterial activity and ligand and Mn (II) complex were in next levels.

Keywords: *Cinnamomum zeylanicum,* NI, metal complex, cytotoxic activity, antioxidant activity, antibacterial activity.

Introduction

People use medicinal plants for many years as a treatment of aliments. The practice of traditional medicine is still strong in treatment infection diseases. Plant-derived compounds offer sources for new anticancer, antioxidant, antibiotics and anti HIV agent [1, 2]. All plants have secondary metabolites which include steroids, alkaloids, flavonoids, tannins and phenol compounds. Recently, investigating active compounds is important for finding new ways for cure of different diseases. The genus *Cinnamomum* belonging to the family Lauraceae. *Cinnamomum zeylanicum* is a medicinal plant, it grows up to 30 feet high, has shiny and smooth twin ovate oblong leaves, large seeds and silky flowers. In previous investigation, the essential oil of the stem bark has two phenylpropanoids, cinnemaldehyde (65 to 75%), eugnol (5 to10%) and other compounds. This plant has different biological activity such as anticancer, antioxidant, antiviral, antibacterial and antifungal activities [3-6]. Presence of these bioactive compounds causes some biological effects. In the present work, we investigated cytotoxic, antioxidant and antibacterial effects of the ligand and metal complexes synthesized from herb of *C.zeylanicum*. The ligand abbreviated as NI that contained cinnemaldehyde (essential oil of the stem bark) and 4-Pyridinecarboxylic acid hydrazide. The Fe (III) and Mn (II) complexes of this ligand prepared by reaction chloride salts of Fe (III) and Mn (II) with NI in dry acetonitrle. After we synthesized these compounds we investigated biological effects.

Materials and Methods

Chemicals and Reagents

4-Pyridinecarboxylic acid hydrazide, Iron (III) chloride hexahydrate and manganese chloride, were Merck chemicals and were used without further purification. Organic solvents were reagent grade. Electronic spectra were recorded by Camspec UV–Visible spectrophotometer model Wpa bio Wave S2 100. The IR spectra were recorded using FT-IR Bruker tensor 27 spectrometer. ^{1}H NMR and ^{13}C-NMR were recorded on a Bruker AVANCE DRX 500 spectrometer at 500 and 125 MHz respectively. All the chemical shifts are quoted in ppm using the high-frequency positive convention; ^{1}H and ^{13}C-NMR spectra were referenced to external $SiMe_4$. The percent composition of elements was obtained from the Microanalytical Laboratories, Department of Chemistry, OIRC, Tehran.

Cell culture

The ligand and metal complexes were assayed for cytotoxicity in vitro against K562 cell line: human chronic myeloid Leukemia (Pasteur, C122), Jurkat cell line: human T Lymphocyte carcinoma (Pasteur, C121) and $T_{47}D$ cell line: human Breast cancer (Pasteur, C203). The three cell lines were provided by the Pasteur Institute Laboratory of natural and Biomimetic in Iran. These cells were grown at 37 °C in an atmosphere containing 5% CO_2, with RPMI-1640 (Gibco, No 51800-019) medium with L-glutamine and 25 mM HEPES (Sigma-Aldrich Chemie GmbH, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, USA), 2.7% sodium bicarbonate and 100 U/mL penicillin and 100 μg/mL streptomycin [7, 8].

Experimental

Synthesis of the ligand

To a magnetically stirred mixture of 4-Pyridinecarboxylic acid hydrazide (1.37 g, 10 mmol) in hot methanol (20 ml) was added to the cinnamaldehyde (1.32 ,1mmol) via a syringe and heated for 45 min at 60°C. After cooling to room temperature, the resulting yellow precipitate was filtered and washed with hexane (20 mL) (Fig. 1).

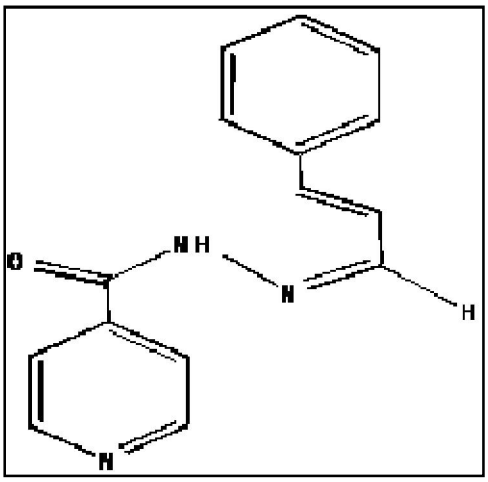

Fig.1. Structure of the ligand, NI

Analysis of the ligand

The analytical and physical data of the ligand are given in follow: Yellow crystals (1.84 g, 92%). Mp 200–203.3°C. IR (KBr) (υ_{max}, cm^{-1}): 3233 (N-H), 1676(C=O), 1640 (C=N), 1451 (C=C), 1308 (C-N), 1174 (N-N). ^1H NMR ($CDCl_3$, Me_4Si): δ_H 6.06 (1H, t, CHC), 6.94 (1H, d, NCH), 7.25 and 7.44 (5H, 2q, $^3J_{HH}$=2.12, 5.28, arom), 7.51(1H, q, $^3J_{HH}$ =15.97, CHCH), 8.50 and 9.21 (4H, 2d, $^3J_{HH}$ =7.83, pyridine) . ^{13}C NMR ($CDCl_3$, Me_4Si): δ_C 124.76 (C_{13}), 127, 128.92, 129.14 and 135.81 (arom), 130.42 (C_{12}), 144.62 (C_{11}), 122.83, 143.55 and 151.06 (pyridine), 165.05 (OCN).Anal. calcd for $C_{15}H_{13}N_3O$ (251.30): C, 62.70; H, 5.17; N, 16.71%. Found: C, 63.12; H, 5.64; N, 17.07%.

Synthesis of the metal complexes; General Method

A solution of metal salt dissolved in acetonitrile added gradually to a stirred acetonitrile solution of the ligand (NI), in the molar ratio 1:1 (metal: ligand). The reaction mixture was further stirred for 2-3h to ensure of the completing and precipitation of the formed complexes. The precipitated solid complexes were filtered, washed several times with 50% (v/v) ethanol–water to remove any traces of the unreacted starting materials. Finally, the complexes were washed with diethyl ether and dried in vacuum desiccators over anhydrous $CaCl_2$.

Analysis of $[Fe(C_{15}H_{12}N_3O)]Cl_2$ (NIFC)

Dark brown crystals. Yield 82%. IR absorptions (cm^{-1} KBr): 1673 ν(C=N), 1622 ν(C=O), 1158 ν(C=C), 1298 ν(C-N), 1137 ν(N-N), 611-997 ν(C-H), 474 ν(Fe-N) and 458 ν(Fe-O). UV- vis (MeCN): λ_{max} 314 nm (ε 260), λ 357nm (ε 156).

Analysis of $[Mn(C_{15}H_{12}N_3O)]Cl_2$ (NIMC)

Dark yellow crystals. Yield 95%. IR absorptions (cm^{-1} KBr): 1669 ν(C=N), 1632 ν(C=O), 1543 ν(C=C), 1310 ν(C-N), 1139 ν(N-N), 683-992 ν(C-H), 508.6 ν(Mn-N), 455 ν(Mn-O). UV- vis (MeCN): λ_{max} 320 nm (ε 200).

Biological studies

Cytotoxicity assay

Cells were washed by PBS (phosphate buffer saline) and they were plated at a density of 1^x-10^4 cell/well in 200 μL medium, into 96-well plates. These plates were maintained for 24h at 37°C in a ancubator, then the medium was changed and the cells were treated with media containing different concentration of compounds (25, 50, 100, 200 μg/mL) dissolved in sterile DMSO. The dilution medium without the sample, used as a control, next plates were incubated for time periods of 24, 48, 72h the cytoxic effect of the ligand and complexes on three human cancer cell lines were assayed by the MTT assay. Cell viability assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is based on the mitochondrial enzyme reduction of tetrazolium dye to determine cell viability. After the incubation time, MTT solution (2.5 mg/mL) was added to each well [9,10]. After 2-4h incubation, the medium was removed and 200 μL of DMSO were added to each well. After shaking for 5 miniuts, the content of the wells was homogenized. The absorbances were measured on a microplate ELISA reader at 660 nm [9-11]. The IC_{50} value was the compound concentration needed for killing 50% of the tumor cells that were defined by comparsion of the number of tumor cells in test plates and control plate.

Viability percentage was evaluated as $OD_{TREATMENT}/OD_{CONTROL}$ [1, 12].

Morphological observation

The morphology of K562, Jurkat and $T_{47}D$ cells was observed initially by inverted microscope before performing experiments. Apoptotic morphology of them was further observed by acridine orange/ethidium bromide (AO/EB) double staining. The cell lines were seeded at a density of $5–10^5$ cells/well and $2.5\text{-}10^5$ cells into 25 cm^2 flask, respectively. The cells were incubated without or with different concentration of the extracts (15 and 20 µg/mL) after 24h adherence. Following 24 and 48h incubation, cells were washed with PBS and harvested by trypsine /EDTA. They were resuspended in culture medium at a density of $1\text{-}10^7$ cells /mL. The cells were stained with 0.1 mg/mL AO and 0.1 mg/mL EB (Sigma) in PBS, then immediately examined under a fluorescence photomicroscope [12].

DPPH radical scavenging activity

The antioxidant activity determined by DPPH. Serial dilutions were prepared with the stock solutions (1 mg/mL) of compounds to obtain concentrations 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078, 0.0039 and 0.019 mg/mL. Methanol was solvent for all the solutions. Diluted solutions (5 mL each) were mixed with 5 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma). The samples were kept at room temperature for 30 minutes for any reaction to accure. The DPPH radical scavenging activity was determined by measuring the absorbance at 517nm using a spectrophotometer. The RC_{50} value, which is the concentration of the test compounds that reduced 50% of free radical concentration, was calculated as milligram per milliliter [5, 7, 13].

Antibacterial Assays

The antibacterial activities of compounds were determined against *Esherchia coli* (PTTC 1047), *Bacillus cereus* (PTTC 1247), *Bacillus pumillus* (PTTC 1319), *Erwinia carotovora* (PTTC 1675), *Staphylococcus epidermidis* (PTTC 1114), *Staphylococcus aureus* (PTTC 1112), by agar disc diffusion method and agar dilution method. All the tests were performed in muller hinton agar (MHA) for preparation of the media.

Disc diffusion method

Disc diffusion methods were employed for evaluation of antibacterial activity of tested compounds. The compounds were dissolved in dimethyl sulfoxide (DMSO). The filter paper discs (6 mm in diameter) were individually impregnated with 30 µL of stock solution of the compounds (10 mg/mL), which had been previously seeded with the tested microorganism. The inoculated plates were incubated at 37°C for 24h [11, 14]. After incubation, plates were observed for zone of inhibition stock solution of the compounds. Antibacterial activity was evaluted by measuring the distance of inhibition zones, that were expressed in millimeters [1, 7, 15]. Two different available antibiotic discs, Nitroforantoine (300 mcg per disc) and Gentamicine (10 mcg per disc) were used as positive control.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the compounds against the tested bacterial was determined by the agar dilution method. The concentrations of each tested compounds incorporated in MHA were 1.5, 3, 6, 12.5, 25, 50, 100, 500, 1000, 1500, 2000, 2500 µg/mL. The antibacterial activities were examined after incubation at 37°C for 24h. Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of test samples which resulted in a complete inhibition of visible growth [16].

Results

Preparation for Ligand, NI, Fe (III) and Mn (II) complexes

The reaction of Fe (III) and Mn (II) salts with the ligand, NI, results in the formation of [ML] for M= Fe (III) and Mn (II). All complexes are quite stable and could be stored without any appreciable change. The NI ligand and the $[Fe(C_{15}H_{12}N_3O)]Cl_2$ complex have 200–203.3°C and 199-200°C melting point respectively, but the $[Mn(C_{15}H_{12}N_3O)]Cl_2$ complex do not have sharp melting point but decompose above 200°C. These complexes insoluble in common organic solvents, such as ethanol, methanol, chloroform or acetone. However, they are soluble in DMSO and DMF. Their structures were characterized by elemental analysis, ^1H NMR and IR. Their elemental analyses are in accord with their proposed formula. The spectral data of the complexes have good relationship with the literature data.

Biological Studies

Cytotoxicity studies

The ligand and complexes had been tested against three human cancer cell lines: K562, Jurkat and $T_{47}D$. The general method used for testing on cytotoxic effect of these compounds is the standard method that has been explained in greater detail. The IC_{50} for each

Table-1. 72 hour IC_{50} values (μg/mL) obtained for three compounds

Compound	IC_{50} for cell line		
	K562	Jurkat	$T_{47}D$
NI	109	86.12	165.45
NIFC	86.12	77.86	118.52
NIMC	82	78.90	181.43

Table 2. Antimicrobial activity of the complexes of *C.zeylanicum* using disc diffusion (mm) and agar dilution methods (MIC, mg/mL)

Extract / Bacteria	Fe (III) complex		Mn(II) complex		Ligand		AB	
	Disc Diffusion	MIC	Disc Diffusion	MIC	Disc Diffusion	MIC	FM	GM
E.coli	13	0.05	—	—	12	0.1	17	15
E.carotovora	28	0.0015	15	0.006	14	0.0015	24	20
S.epidermidis	15	0.0125	—	0.05	—	0.025	0	11
	19	0.0125	12	—	14	0.05	25	28
S.aureus	20	0.006	40	0.05	14	0.003	18	22
B.cereus	18	0.025	11	—	16	0.05	30	26
B.pumillus								

compound was measured by using MTT. The IC_{50} values are shown in Table1 [17, 18].

MTT assay indicated that various concentrations of these compounds decrease the viability of these cell lines. Morphological observations of K562, Jurkat and $T_{47}D$ were performed by acridine orange/ethidium bromide (AO/EB) double staining. According to our results it seems that compounds act via induce of apoptotic death on cancer cells. We suggest that concentrations of the compounds cause programmed cell death.

The DPPH assay showed that Mn (II) complex have the highest antioxidant activity and could scavenge free radicals with RC_{50} value of 0.394 mg/mL, Fe (III) complex and ligand are in the next levels with RC_{50} value of 0.724 and 1.429 mg/mL, respectively.These compounds have weak antioxidant activity. The antibacterial activities of the compounds were determined against gram-negative and gram-positive bacteria by agar disc diffusion and agar dilution method. The results are summarized in Table 2.

The data showed that Fe (III) complex has the highest antibacterial activity, the ligand and Mn (II) Complex are in next levels.

Discussion

The ligand and metal complexes of *C.zeylanicum* had cytotoxic, antioxidant and antibacterial activities. These biological properties are due to cinnamaldehyde and structure of synthetic hetrocycle. According to the above sentences, these compounds offered as a new outlook for chemotherapy. The results showed that the ligand and metal complexes exhibit high cytotoxic potential on these cell lines. The mechanism by which these compounds act as antitumor agent is apoptosis, indicated in morphological studies. Probably present of cinnamaldehyde which lead to apoptosis. It has also been proposed that concentration plays a vital role in increasing the degree of inhibition. Antioxidant potential is probably because of the presence of cinnamaldehyde in these compounds. It is important to note that the metal complexes show intensified

antioxidant activity compared to the parent ligand. Antibacterial properties of new compounds had been studied and good results were obtained. These compounds can be useful for supply antibiotics. There for, it is suggested that further work be performed on the other metal complexes of *C.zeylanicum* herbs. The result showed that the ligand and its complexes could be considered as a plant-derived chemotropic, antioxidant and antibiotics agents alike [1, 17-20].

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