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Thermal Analysis, Antioxidant and *in vitro* Antimicrobial Activity of Palladium(II) Complexes with N,N'- Ethylenediamine Bidentate Ester Ligands

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ABSTRACT

Palladium (II) complexes (1-4) with bidentate N,N'-ligands, O,O'-dialkyl esters (R=ethyl, n-propyl, n-butyl and n-pentyl), of (S,S)-ethylenediamine-N,N'-di-2-(4-methyl)pentanoic acid (S,S-eddp) (L1•2HCl-L4•2HCl) were prepared. The ligands and its complexes were tested for their in vitro antimicrobial and antioxidant activities. The antimicrobial activity was tested using broth dilution procedure for determination of the minimum inhibitory concentration (MIC). The MICs were determined for 6 selected indicator strains. The Pd (II) complex with the n -Pe group in ester chain displayed the strongest antimicrobial activity among the all tested compounds. The results showed that the ligand with the n -Bu group in ester chain possessed the highest total antioxidant activity and antioxidant activity at the level of the DPPH and ABTS radicals. The IC_{50} values determined were: 12.89 ± 1.75 to 8.29 ± 0.64 µg/mL for DPPH free radical scavenging activity and 22.46 ± 1.68 to 14.46 ± 1.68 for ABTS radical scavenging assay. Also, the results of thermal decomposition have been discussed.

Keywords: Palladium(II) complexes, Antimicrobial activity, Antioxidant activity, Thermal analysis

INTRODUCTION

Throughout the twentieth century, the development of antimicrobial medicines was claimed to be one of the most successful events. Resistance against antimicrobial agents has developed as a vital public health problem worldwide [1-5]. During the process of searching for the innovative drugs against the drug-resistant diseases, very close attention was paid to the usage of metal complexes [6-13], thus resulting in a variety of exciting and invaluable drugs such as cis-platin [13]. Research is being conducted in fields such as cancer [14,15], diabetes [16-18], arthritis [19], magnetic resonance imaging [20], metal-mediated antibiotics, antibacterial, antiviral, antiparasitic, and radiosensitizers [21-24].

The field which draws interest of many researchers is the synthesis and evaluation of biological activity of the new metal-based ingredients. Many palladium (II) complexes have been synthesized and their biological activities have been announced [25-27]. Studies were conducted on the impact of different palladium complexes on the growth and metabolism of various groups of microorganisms. Garoufis et al. [21] reviewed numerous scientific research on antiviral, antibacterial and antifungal activity of palladium (II) complexes with different types of ligands (sulfur and nitrogendonor ligands, Schiff base ligands and drugs as ligands). Guerra et al. [28] synthesized three palladium complexes with antibiotics of the tetracycline family and they tested their effects on tetracycline sensitive and resistant bacterial strains. The palladium complex with tetracycline was 16 times stronger than the tetracycline itself against resistant strain. Vi Palladium (Ii) complexes, Antimicrobial activity, Antioxidant activity, Thermal analysis eira et al. [29] prepared new palladium(II) and platinum(II) complexes with fluoroquinolones which showed activity to Mycobacterium tuberculosis. There are other papers in the literature showing different intensity of palladium complexes activity on various species of bacteria and fungi [30-35].

Synthesis and characterization of R_2edda-type esters (edda=ion of ethylene-diamine-N, N'-diacetic acid) and the

corresponding platinum(II/IV) and palladium(II) complexes was reported [36-40]. In our previous articles, we have investigated the synthesis, and characterization of palladium(II) complexes (1-4), their *in vitro* cytotoxic activity against chronic lymphocytic leukemia cells (CLL), human breast cancer (MDA-MB-361, MDA-MB-453), T leukemia (Jurkat) and chronic myelogenous leukaemia (K562) cell lines, and they were compared to the activity of ligand precursors L1•2HCl-L4•2HCl. *In vitro* activity is increasing in following order L•2HCl ≤ [$PdLCl_2$] [39, 40].

Also, antimicrobial activity and thermal analysis of Cr(III) complexes with edda-type ligands published earlier [41].

Here, we present evaluation of the *in vitro* antimicrobial and antioxidant activities and thermal analysis of four R_2edda-type ligand precursors: O,O'-diethyl-(L1•2HCl) O,O'-dipropyl-(L2•2HCl) O,O'-dibutyl-(L3•2HCl) O,O'-dipentyl-(S,S)-ethylenediamine-N,N'-di-2-(4-methyl)-pentanoate dihydrochlorides (L4•2HCl) and their corresponding palladium(II) complexes: dichloro(O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(4-methyl)-pentanoate) palladium(II), (1), dichloro(O,O'-dipropyl-(S,S)-ethylenediamine-N,N'-di-2-(4-methyl)pentanoate) palladium (II) (2), dichloro(O,O'-dibutyl-(S,S)-ethylenediamine-N,N'-di-2-(4-methyl)-pentanoate)palladium (II) (3), dichloro(O,O'-dipentyl-(S,S)-ethylenediamine-N,N'-di-2-(4-methyl)-pentanoate)palladium(II) (4).

MATERIALS AND METHODS

Complexes and solutions

All the ligands L1•2HCl-L4•2HCl and corresponding palladium (II) complexes (1-4) were prepared using appropriate modifications of known methods [36-39]. Synthesized esters L1•2HCl-L4•2HCl and corresponding palladium (II) complexes (1-4) shown in **Figure 1**. The structure and purity of the samples was confirmed by ^1H and ^{13}C NMR spectroscopy; ^1H and ^{13}C NMR spectra were recorded by a Varian "Gemini 2000" (200 MHz) spectrometer in $CDCl_3$ using tetramethylsilane as internal standard. 1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), Folin-Ciocalteu, Muller-Hinton broth, ascorbic acid (AA), gallic acid (GA) butylated hydroxytoluene (BHT), nystatin, amracin, DMSO and pyrocatechol were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and reagents were of analytical reagent grade.

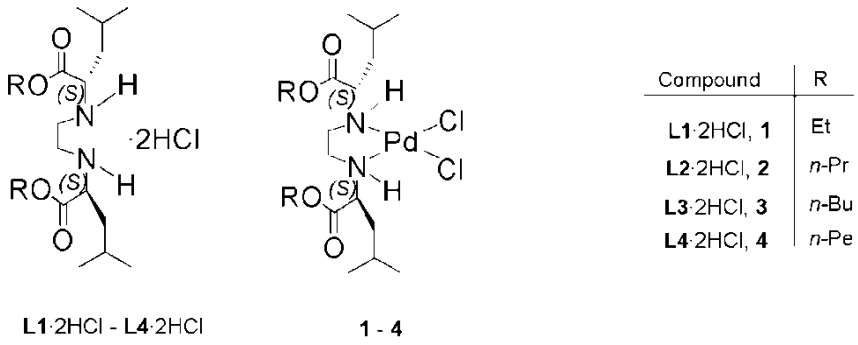

Figure 1: Synthesized esters L1•2HCl–L4•2HCl and corresponding palladium(II) complexes (1-4).

Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV-Vis spectrophotometer MA9524-Spekol 211 (Iskra-Carl Zeiss, Slovenia).

Test microorganisms

In vitro antimicrobial activity of the ligands L1•2HCl-L4•2HCl and corresponding Pd(II) complexes (1-4) was evaluated against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10231 using antibiotics amracin and nystatin are the standards. The fungi were cultured on potato-glucose agar for 7 days at room temperature of 20ºC under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of the samples against the test bacteria were determined by the microdilution method in 96 multi-well microtiter plates [42]. All tests were performed in Muller-Hinton broth (MHB) with yeast being the exception to this, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of samples (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) was pipetted into the first row of the plate. 50 µL of Mueller-Hinton or Sabouraud dextrose broth (replaced by Tween 80 at a final concentration of 0.5% (v/v) for analysis of samples) was added to the other wells. A volume of 50 µL from the first test wells was pipetted into the second well of each microliter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. 10 µL of resazurin indicator solution (prepared by dissolution of a 270 mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas nystatin was used as the control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 24 h for the bacteria and at 28°C for 48 h for the yeast. Subsequently, the color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which a change in color occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard medicine.

Determination of total antioxidant activity

The total antioxidant activity of the samples was determined based on the phosphomolybdenum method [43]. The assay is based on the reduction of Mo (VI)-Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo(V) complex at acid pH. A total of 0.3 mL of sample (in 10% DMSO) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm with a spectrophotometer against the blank after cooling down to room temperature. 10% DMSO (0.3mL) was used as the blank instead of the samples. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of complex/ligand.

Determination of DPPH free radical scavenging activity

The method used by Takao [44] was adopted with suitable modifications from Kumarasamy [45]. DPPH (2,2-dephenyl-1-picrylhydrazyl) (8 mg) was dissolved in 10% DMSO (100 mL) to keep a concentration of 80 µg/mL. Serial dilutions were performed with the stock solution (1 mg/mL) of the samples. Solutions (2 mL each) were afterwards mixed with DPPH (2 mL) and let stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in 10% DMSO to result in making the stock solution with the same concentration (1 mg/mL). The control sample was made using the same volume without test compounds or reference antioxidants. 10% DMSO was used as a blank. The DPPH free radical scavenging activity (%) was calculated through the following equation:

$$\% \text{ inhibition} = Ac\text{-}As/Ac \times 100 \quad (1)$$

The IC_{50} value, described as the concentration of the test material that leads to a reduction of 50% in the free radical concentration, was calculated as µg/mL through a sigmoidal dose-response curve.

ABTS radical scavenging assay

The antioxidant capacity was estimated in terms of the ABTS•+ radical (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity following the procedure described by Delgado-Andrade et al. [46]. Briefly, ABTS•+ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The ABTS•+solution (stable for 2 days) were diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance at 730 nm of 0.70 ± 0.02. After the addition of 10 µL of sample to 4 mL of the diluted ABTS•+ solution, the absorbance was measured at 30 min. Gallic acid (GA), ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as reference antioxidants. All samples were analyzed in triplicate.

Thermal analysis

Thermal analysis was performed with a Simultaneous Thermal Analyzer STA 449 C with identification of the final products by X-ray powder diffraction. In the present investigation the heating rates were suitably controlled at 5°C min-1 under argon atmosphere and the mass loss was measured from ambient temperature up to 1000°C.

RESULTS AND DISCUSSION

Antimicrobial activity

The minimum inhibitory concentration (MIC) values of the ligands L1•2HCl-L4•2HCl and corresponding Pd (II) complexes (1-4) are given in **Table 1**. MICs were determined for six selected indicator strains. Tested palladium complexes and ligands showed different degrees of antimicrobial activity compared to the tested species of microorganisms. The intensity of the antimicrobial activity varies depending on the strains of the microorganisms and the type of the compound. The results show antimicrobial activity specific microdilution method palladium complexes and ligands in the concentration range from 39.1 mg/mL to 312.5 mg/mL, which is generally a good to moderate antimicrobial activity compared to standard antibiotic amracin (for bacteria) and nystatin (for fungi).

Table 1: Minimum inhibitory concentration (MIC, μg/mL) of the ligands (L1•2HCl-L4•2HCl) and complexes (1-4).

Microbial strains	L1	L2	L3	L4	1	2	3	4	A[a]	N[b]
Staphylococcus aureus ATCC 25923	156.25	78.125	78.125	78.125	156.25	156.25	78.125	78.125	0.97	/
Klebsiella pneumonia ATCC 13883	156.25	312.5	312.5	156.25	78.125	156.25	78.125	39.1	0.49	/
Proteus vulgaris ATCC 13315	156.25	156.25	312.5	312.5	156.25	78.125	156.25	78.125	0.49	/
Proteus mirabilis ATCC 14153	156.25	78.125	78.125	78.125	156.25	312.5	312.5	156.25	0.49	/
Bacillus subtilis ATCC 6633	312.5	312.5	312.5	312.5	78.125	78.125	156.25	39.1	0.24	/
Candida albicans ATCC 10231	78.125	78.125	39.1	156.25	39.1	312.5	78.125	78.125	/	1.95

[a] Amracin
[b] Nystatin

Ligand L1 showed equally good activity against all tested bacteria at a concentration of 156.25 mg/mL, except for the bacteria *B. subtilis* (MIC=312.5 mg/mL). Very good activity showed the fungus *C. albicans* at a concentration of 78.125 mg/mL. The bacteria *S. aureus* and *P. mirabilis* and fungus *C. albicans* are the strongest sensitivity exhibited towards ligand L2 at a concentration of 78,125 mg/mL, while the lower sensitivity of *P. vulgaris* exhibited (MIC=156.25 mg/mL) and the lowest sensitivity *K. pneumoniae* and *B. subtilis* at a concentration of 312.5 mg/mL. The strongest activity against *S. aureus* and *P. mirabilis* bacteria (MIC=78.125 mg/mL) and the fungus *C. albicans* (MIC=39.1 mg/mL) showed ligand L3. *K.pneumoniae*, *P.vulgaris* and *B. subtilis* are less sensitive to the Ligand L3 and at a concentration of 312.5 mg/mL. Also, the strongest sensitivity exhibited the bacteria *S. aureus* and *P. mirabilis* (MIC=78.125 mg/mL) to the ligand L4. Slightly less activity ligand L4 was expressed to the fungus *C. albicans* and *K. pneumonia* bacteria at a concentration of 156.25 mg/mL and the least susceptible bacteria *P. vulgaris* and *B. subtilis* (MIC=312.5 mg/mL).

The complex 1 showed the strongest activity against the fungus *C. albicans* (MIC=39.1 mg/mL), somewhat weaker against bacteria *B. subtilis* and *K. pneumoniae* (MIC=78.125 mg/mL), while the lowest activity showed to the bacteria *S. aureus*, *P.vulgaris* and *P. mirabilis* in a concentration of 156.25 mg/mL. Moderate activity showed a complex 2 the bacteria *B. subtilis* and *P.*vulgaris (MIC=78.125 mg/mL) and showed weaker activity against other strains tested. The complex 3 are most susceptible *C. albicans* fungi and bacteria *B. subtilis* and *K. pneumoniae* at a concentration of 78,125 mg/mL, while somewhat lower sensitivity exhibited *P. vulgaris* and *B. subtilis* (MIC=156.25 mg/mL). The lowest sensitivity was expressed in *P. mirabilis* bacteria concentration of 312.5 mg/mL at complex 3. The complex 4 showed very strong activity against bacteria *K. pneumoniae* and *B. subtilis* at a concentration of 39.1 mg/mL, and P.vulgaris against bacteria and fungus *C. albicans* and *S. aureus* (MIC=78.125 mg/mL), while the lowest activity exhibited by *P. mirabilis* bacteria in a concentration of 156.25 mg/mL.

In general, complexes of Pd (II) are more active than their ligands. Comparing the activity of the ligands and the

corresponding complexes, it was found that the complexes 1 and 4 significantly more active than the corresponding ligands, unlike the complex 2 and 3 where the difference between them and the corresponding ligands insignificant.

Due to the obtained results, the higher activity of the complexes, as compared to the ligands, can be understood in terms of chelation theory. This theory explains that a decrease in the polarizability of the palladium (II) ion could enhance the lipophilicity of the complexes [47].

If we observe the ligands between themselves, ligands L2 and L3 are slightly stronger antimicrobial activity for the majority of the strains compared to ligands L1 and L4. However, if we observe the complexes between themselves, complexes 1 and 4 have a stronger antimicrobial activity compared to complexes 2 and 3. The complex 4 displayed the strongest activity among the all tested compounds.

Most resistant bacteria to ligands were *B. subtilis*, a most sensitive *S. aureus* bacteria and fungus *C. albicans*. *P. mirabilis* bacteria as manifested most resistant to the effect of Pd (II) complexes, while all other strains were sensitive. When compared with the control antibiotic see that the complexes showed good antimicrobial activity, while the ligands showed moderate activity.

Antioxidant activity

Table 2 shows that a slightly higher total antioxidant power showed ligands relative to their respective complexes. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of ligands/complex. Better overall antioxidant activity show all ligands of their complexes. The results of the total antioxidant activity in comparison with the results of antioxidant activity in DPPH and ABTS level radicals. The highest total antioxidant activity shows L3 which also has the highest power of binding DPPH and ABTS radicals. IC_{50} values of antioxidant activity at the level of the DPPH and ABTS radicals of Pd (II) complexes and ligands were reported in **Table 2**. IC_{50} values were determined for each measurement: 11.45 ± 1.55 (L1), 10.45 ± 1.15 (L2), 8.29 ± 0.64 (L3), 10.18 ± 1.05 (L4), 12.89 ± 1.75 (1), 11.45 ± 1.55 (2), 10.29 ± 0.64 (3) and 11.18 ± 1.05 (4) μg/mL for DPPH free radical scavenging and 16.89 ± 1.55 (L1), 15.23 ± 1.45 (L2), 14.46 ± 1.68 (L3), 16.04 ± 0.78 (L4), 22.46 ± 1.68 (1), 20.46 ± 1.68 (2), 17.23 ± 1.45 (3), 19.34 ± 0.78 (4) for ABTS radical scavenging assay **(Table 2)**. Also, it is noted that there was no significant difference in the results for the antioxidant activity as ligands and complexes. The best antioxidant activity and have demonstrated complexes and their corresponding ligands on level of DPPH radicals.

Table 2: Antioxidant activity of the ligands (L1•2HCl-L4•2HCl) and complexes (1-4).

Sample	DPPH scavenging activity [a]IC_{50} (μg/ml)	ABTS radical scavenging assay [a]IC_{50} (μg/ml)	Total antioxidant capacity (μg AA/g)
L1	11.45 ± 1.55*	16.89 ± 1.55	98.45 ± 0.98
L2	10.45 ± 1.15	15.23 ± 1.45	101.45 ± 0.98
L3	8.29 ± 0.64	14.46 ± 1.68	123.75 ± 0.25
L4	10.18 ± 1.05	16.04 ± 0.78	99.34 ± 0.43
1	12.89 ± 1.75	22.46 ± 1.68	79.94 ± 0.54
2	11.45 ± 1.55	20.46 ± 1.68	78.45 ± 0.98
3	10.29 ± 0.64	17.23 ± 1.45	69.75 ± 0.25
4	11.18 ± 1.05	19.34 ± 0.78	96.34 ± 0.43
Gallic acid	3.79 ± 0.69	1.96 ± 0.41	-
Ascorbic acid	6.05 ± 0.34	10.98 ± 0.95	-
BHT	15.61 ± 1.26	7.23 ± 0.87	-
α-Tocopherol	-	0.48 ± 0.05	-

[a]IC_{50} values were determined by nonlinear regression analysis.
*Results are mean values ± SD from three experiments.

The stable DPPH radical scavenging is a widely used method to evaluate antioxidant activities, in a relatively short time compared with other methods. The DPPH radical contains an odd electron responsible for absorbance at 517 nm. When the reaction between antioxidant molecules and DPPH radical results in the scavenging of the radical by hydrogen or electron donation, the antioxidants cause to a decrease of the absorbance of the DPPH radical. This is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the activity of antioxidants [48].

There were no significant differences in the activities of the complex and the corresponding ligands on the antioxidant

activity. Ligands themselves showed higher antioxidant activity than the corresponding complexes. Also, all the results showed comparable or slightly lower activity compared to the corresponding standards (Gallic acid, ascorbic acid and BHT).

Thermal analysis

The metal complexes under investigation have very similar thermal behavior in the temperature range 20-1000°C.

All the Pd (II) complexes are stable up to 160°C and then decompose in two major steps **(Table 3)**. Degradation of these complexes begins by the dehalogenation overlapping with the ligand decomposition and isn't influenced by the type of the complex. The temperature range for the first endothermic step is 160-230°C for the Pd (II) complexes where losses of mixed fragments occur. In the range 290-730ºC one very broad peak is present in the DTA curves of the complexes, and the ligands' fragments could not be approximated due to continuous mass loss. There is the maximum mass loss (78.5-81.5%) as a result of the dehalogenation and the decomposition of the ligand molecules in the temperature range 210-450ºC **(Table 3)**. In **Figure 2** is shown TG diagram for Pd (II) complexe with the η -Bu group in ester chain, 3, as typical for these complexes.

Table 3: Thermal analysis data for decomposition of the Pd (II) complexes (1-4).

Complex	Ranges of decomposition (°C)	Mass loss, %		Assignment	ΔH (kJ mol^{-1})
		Found	Calc.		
1	195-215 300-730	78.5	79.6	Dehalogenation and decomposition of the ligand molecules	73.73
2	160-230 290-700	80.3	80.6	Dehalogenation and decomposition of the ligand molecules	74.86
3	220-225 285-745	80.4	81.6	Dehalogenation and decomposition of the ligand molecules	76.46
4	205-230 300-675	81.5	82.4	Dehalogenation and decomposition of the ligand molecules	77.71

The pyrolytic residue is elemental palladium for all the Pd (II) complexes, as was identified by X-ray powder diffraction [49].

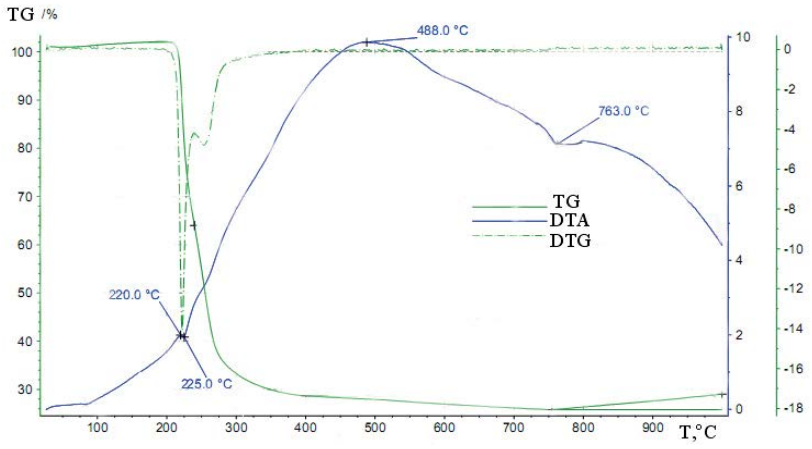

Figure 2: TG diagram for Pd(II) complex 3

CONCLUSION

A series of Pd (II) complexes with bidentate N,N'-ligands, O,O'-dialkyl esters (R=ethyl, η -propyl, η -butyl and η -pentyl), of (S,S)-ethylenediamine-N,N'-di-2-(4-methyl)pentanoic acid (S,S-eddp) (L1•2HCl-L4•2HCl) were prepared. The ligands and its complexes were tested for their *in vitro* antimicrobial activity. Complexes of Pd (II) are more active than their ligands. The complex 4 displayed the strongest antimicrobial activity among the all tested compounds. Ligands themselves showed higher antioxidant activity than the corresponding complexes. Also, all the results showed comparable or slightly lower activity compared to the corresponding standards (Gallic acid, ascorbic

acid and BHT). The results showed that the ligand 3 possessed the highest total antioxidant activity and antioxidant activity at the level of the DPPH and ABTS radicals.

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