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Study of the Catecholase Activity of new catalysts Based on Copper (II) and Heterocyclic Ligands

Rafika El Ati[1], *Haytham Bouammali*[1], *Mohamed El Kodadi*[1,2], *El Bekkay Yousfi*[3], *Rachid Touzani*[1],
Belkheir Hammouti[1,4,*]

[1] Laboratory of Applied Chemistry & Environment, Faculty of Sciences, University Mohammed Premier, Oujda, Morocco.
[2] Laboratoire d'Innovation en Sciences, Technologie et Education (LISTE), CRMEF Oriental, Oujda, Morocco.
[3] Higher Institute of Nursing and Health Professions techniques, ISPITS Oujda Morocco.
[4] Ecole des Hautes Etudes d'Ingénierie EHEI, 60000 Oujda Morocco.
Correspondence: E-mail: hammoutib@gmail.com

ABSTRACT

In this work, we are interested in finding new catalysts for catecholase, whose principle is based on the oxidation reaction of catechol to o-quinone. We approached the synthesis of the threes tripod ligands based on pyrazole in a condensation reaction and its characterization by IR, ^{13}C NMR, ^{1}H NMR spectroscopy, then we evaluated the catalytic properties of certain complexes formed in situ to catalyze the oxidation reaction of catechols to o-quinones. The aim is to find the right models to reproduce the catalytic activity of the enzyme (catecholase), we used complexes formed in situ by pyrazole derivatives with Copper salts. Among these complexes, the complex $L_4/Cu(CH_3COO)_2$ showed good catalytic activity of the combination (1ligand/2metal) in MeOH for this reaction, with a speed *Vmax* equal to 69.38 $\mu mol.L^{-1}.min^{-1}$ and a low value of *Km* equal to 0.019 $mol.L^{-1}$. We have demonstrated that the nature of concentration, ligand, solvent, and copper salts, influenced strongly the catecholase activity.

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1. INTRODUCTION

Catechol oxidase (CO), found in plants, bacteria, and fungi, is a copper (II) enzyme that catalyzes, in the presence of oxygen, the oxidation of o-diphenols to o-quinones (**Figure 1**) (Driessen, 1982). The latter self-oxidize and, after polymerization, form a high molecular weight black-brown pigment called melanin, which is a substance responsible for the post-mortem browning of plants and fruits (Homrich *et al*., 2021; Xue *et al*., 2011).

The development of biomimetic oxidation catalysts, involving the Cu(II) as an active metal center (Gamez *et al*., 2001; Punniyamurthy *et al*., 2008), and numerous biomimetic approaches have been devoted to the synthesis of complexes of Cu(II) with various ligands to reproduce the catalytic activity of biological systems containing in their active sites the Cu(II) (El Kodadi *et al*., 2008; Boussalah *et al*., 2009; Zerrouki *et al*., 2011; Bouabdallah *et al*., 2007a; ; Bouabdallah *et al*., 2007b; Toubi *et al*., 2012; Saddik *et al*., 2012; Saddik *et al*., 2012).

In vivo, this melanosis process plays a key role in the primary immune defenses and the protection of plants against pathogens and insect attacks (Jiang *et al*., 2020; Ramsden *et al*., 2014).

Over the past two decades, many studies, including those in our laboratory, have been devoted to copper complexes carrying nitrogenous heterocyclic ligands (pyrazole, pyridine, imidazole, etc.) to mimic the function of catecholase (Boussalah *et al*., 2009; Zerrouki *et al*., 2011; El Kodadi *et al*., 2008; Bouabdallah *et al*., 2007a; Bouabdallah *et al*., 2007b; Mouadili *et al*., 2013). This research has shown that catecholase activity depends on many factors, including the distance between the two metal centers, the redox potential of the copper centers, the pH, the structure of the ligand, and the nature of the solvent (Boussalah *et al*., 2009; Zerrouki *et al*., 2011; El Kodadi *et al*., 2008; Bouabdallah *et al*., 2007a; Mouadili *et al*., 2013; Dey *et al*., 2016; Paralta *et al*., 2010; Mendoza-Quijano *et al*., 2012; Mukherjee *et al*., 2004; Paul *et al*., 2017; Mondal *et al*., 2017; Osorio *et al*., 2012; Camargo *et al*., 2015; Neves *et al*., 2010).

Enzymes are generally protein-like biopolymers formed by the polymerization of amino acids. They act as catalysts for reactions occurring in biological media. These biocatalysts are characterized by the following parameters (Carrea *et al*., 2000).

In this work, we are interested in the study of the catecholase activity of four copper (II) complexes formed in-situ by mixing copper salts [$Cu(CH_3COO)_2$, $CuSO_4$, and $Cu(NO_3)_2$] and ligands based on pyrazole and triazole (**Figure 2**). The objective is to search for catalysts that best reproduce the catalytic activity of the enzyme "Catecholase" under mild conditions.

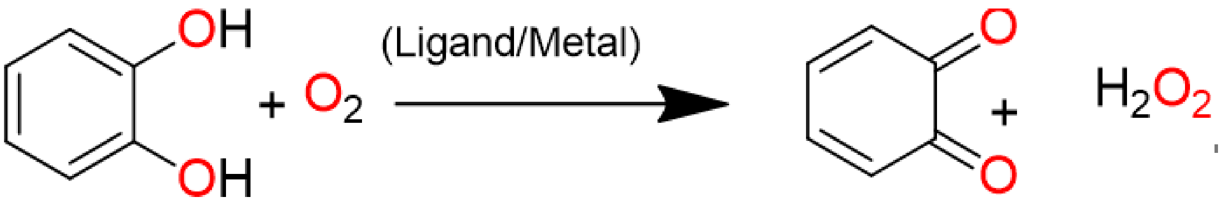

Figure 1. Oxidation of o-diphenols to o-quinones by catechol oxidase.

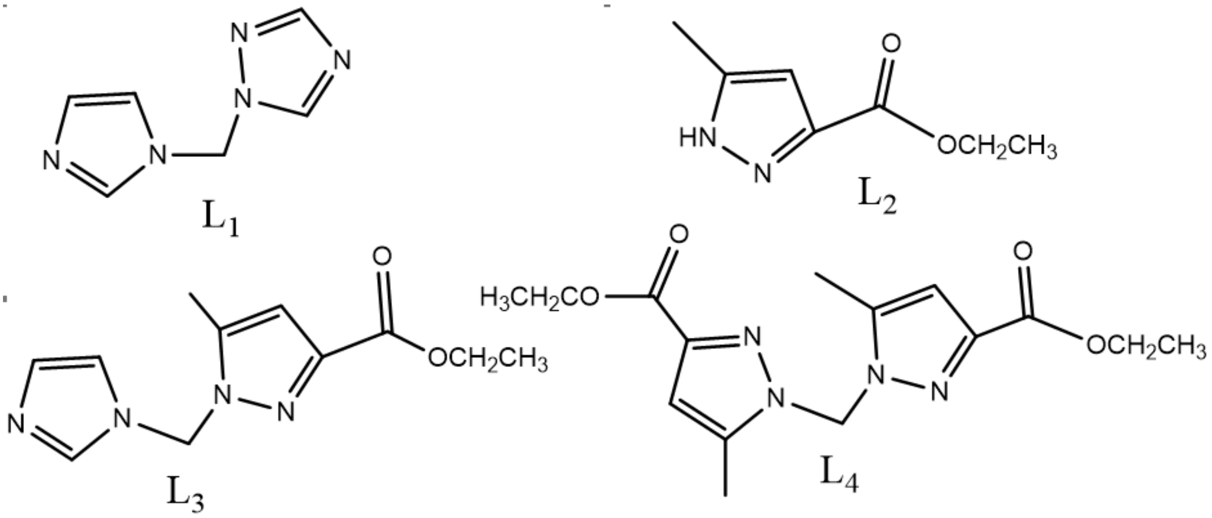

Figure 2. Ligands used in the in-situ formation of Cu (II) complex with catecholase activity.

2. METHOD

2.1. Physical Measurements

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker-400 instrument operating at 400 MHz for ^{1}H spectra and 101 MHz for ^{13}C spectra. Fourier-transform infrared (FT-IR) spectra were recorded on pressed KBr pellets (4500-400 cm-1) using a Shimadzu infrared spectrophotometer. The way how to interpret the NMR and FTIR analysis was explained in the literature (Nandiyanto *et al*., 2019; Gunawan & Nandiyanto, 2021).

2.2. Compounds Synthesis

The compounds **L_1-L_4** (**Figure 1**) were synthesized following the reaction described in the literature (Kaddouri *et al.*, 2020; Abrigach *et al*., 2018; El Kodadi *et al*., 2008; Touzani *et al*., 2011; Hammouti *et al*., 2012; Boussalah *et al*., 2013; Abrigach *et al*., 2016; Abrigach *et al*., 2017; Kaddouri *et al*., 2019; El Ati *et al*., 2019; Bouchal *et al*., 2019), where the L_1 is synthesized by condensation of (4H-1,2,4-triazol-4-yl)methanol with 1H-imidazole, L_2 by condensation diethyl oxalate with propan-2-one (acetone), L_3 is synthesized by condensation of ethyl 1-(hydroxymethyl)-5-methyl-1H-pyrazole-3-carboxylate and 1H-imidazole and L_4 is synthesized by condensation of ethyl 1-(hydroxymethyl)-5-methyl-1H-pyrazole-3-carboxylate with ethyl 5-methyl-1H-pyrazole-3-carboxylate. All the studied compounds were purified either in acetonitrile or dichloromethane to obtain the final products with good yields.

These polydentate ligands **L_1-L_4** provide the two nitrogen sp^2, or one nitrogen sp^2 and one oxygen atom or tow nitrogen sp^2 and one oxygen or tow nitrogen sp^2 and tow oxygen, form the coordinate site capable of coordinating one atom of a metal (Bouabdallah *et al*., 2007b; Toubi *et al*., 2012; Saddik *et al*., 2012) (**Figure 3**).

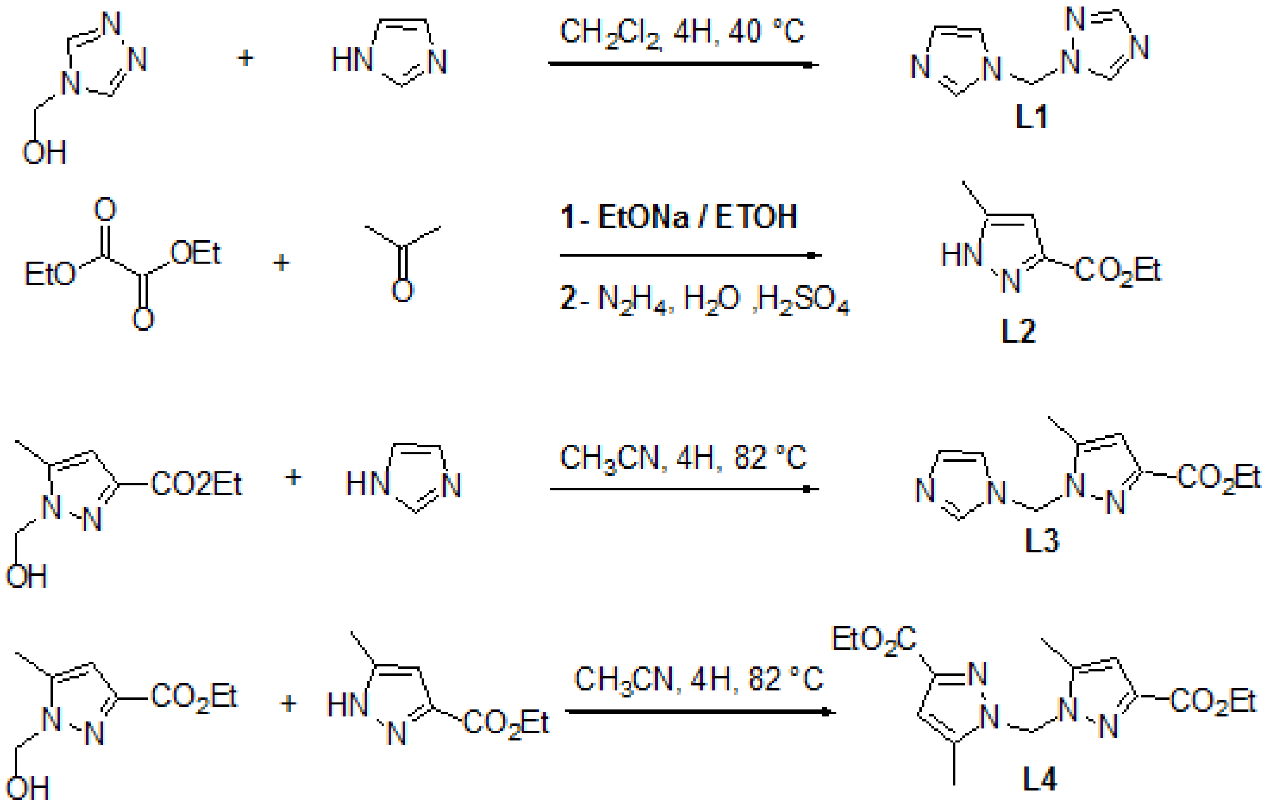

Figure 3. Structure of prepared the heterocyclic ligands **L_1–L_4**.

2.3. Catecholase Activity Measurements

Kinetic measurements were spectrophotometrically made using a JENWAY 7315-Spectrophotometer (ISPITS Oujda), following the appearance of o-quinone overtime at 25°C (390 nm absorbance maximum, $\mathcal{E}$ = 1600 L.mol^{-1}.cm^{-1} in methanol, $\mathcal{E}$ = 1900 L.mol^{-1}.cm^{-1} in THF and DMF). The complexes are formed in situ, by mixing successively: 0.15 mL of a solution, (2.10^{-3} mol.L^{-1}) of metals salts, with 0.15 mL of ligand solution (2.10^{-3}mol.L^{-1}); finally, complexes formed in situ were treated with 2mL (0,25 mol.L^{-1}) of catechol in methanol (MeOH), tetrahydrofuran (THF) or N, N-Diméthylformamide (DMF) under aerobic conditions. The way how to interpret the spectrophotometer was explained in the literature (Pratiwi & Nandiyanto, 2022).

3. RESULTS AND DISCUSSION

3.1. Study of Catecholase in the Presence of Complexes Formed in Situ by Ligands L_1-L_4 with Metallic Salts in the MeOH Solvent

3.1.1. Catalytic study of combinations (1L/1M) in MeOH

Evaluation of the catecholase activity of these complexes was spectrophotometrically made by observing the manifestation of the maximum absorbance of the corresponding o-quinone at 390 nm over time (**Figure 1**). The results are illustrated in **Figure 4**, showing the change in absorbance at 390 nm versus time for 60 min. The obtained spectrum for catechol alone showed practically no absorbance as a function of time.

The change in absorbance as a function of time for the oxidation of catechol in the presence of the complex formed by ligands **L_1-L_4** with copper salts (II) show that the combination formed by ligand **L_3** with $CuSO_4$ is more important than the other combinations such that the oxidation rate reaches an order value of 8.19 µmol.L^{-1}min^{-1}. **Table 1** shows reaction rate V (µmol. L^{-1}.min^{-1}) of catechol oxidation in methanol (1Ligand/1Salt).

According to the results obtained in this **Table 1**, it is noted that all the copper complexes formed in-situ with the ligands **L_1-L_4** catalyze the oxidation reaction of catechol to o-quinone and that the best catecholase activity was recorded with the complex formed from L_3 and the metal salt $CuSO_4$ (8.19 µmol.L^{-1}.min^{-1}).

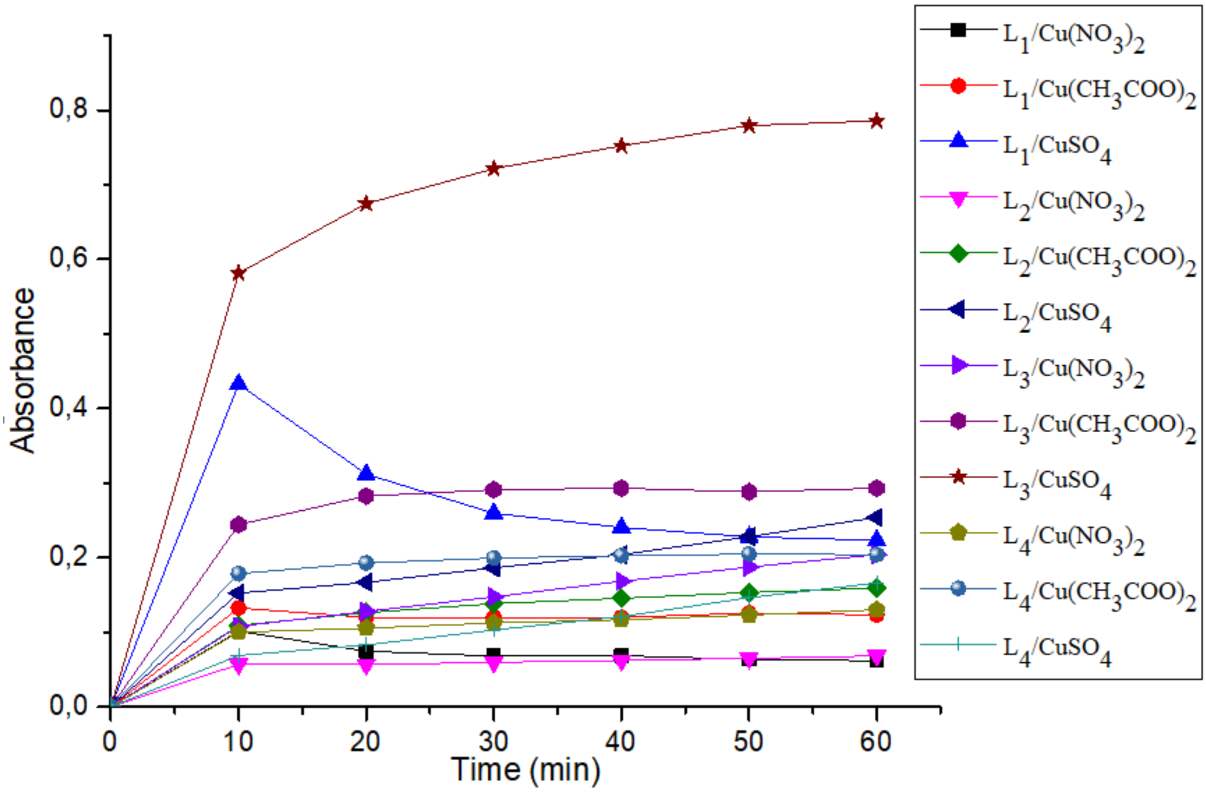

Figure 4. Oxidation of catechol in the presence of complexes formed by L_1 – L_4 and different metals salts (1L/1M) in methanol.

Table 1. Reaction rate V (µmol. L^{-1}.min^{-1}) of catechol oxidation in methanol (1Ligand/1Salt).

L/M	**$Cu(NO_3)_2$**	**$Cu(CH_3CO_2)_2$**	**$CuSO_4$**
L1	1.31	0.64	1.75
L2	0.71	1.66	2.64
L3	2.13	3.05	8.19
L4	1.36	2.12	1.73

3.1.2. Catalytic study of combinations (2L/1M) in MeOH

The catalytic study of the complexes formed in-situ, by successively mixing one equivalent of a solution (2.10^{-3} mol.L^{-1}) of copper salt ($Cu(CH_3COO)_2$, $CuSO_4$ and $Cu(NO_3)_2$) with two equivalents of a solution (2.10^{-3} mol.L^{-1}) of ligand and 2 mL of a catechol solution of 0.25 mol.L^{-1} concentration is added. The spectra of the change in absorbance of o-quinone at 390 nm are shown in **Figure 5** for L_1-L_4, and the oxidation rates for the ligands are collated in **Table 2**.

Catechol oxidation rates in the presence of combinations (2L/1M) based on ligands (L1-L4) and various copper salts (Ⅱ) in MeOH.

According to the results obtained in this **Table 2**, it is noted that all the copper complexes formed in-situ with the ligands **L_1-L_4** catalyze the oxidation reaction of catechol to o-quinone. The best catecholase activity was recorded with the complex formed from $2L_2/Cu(CH_3CO_2)_2$, $2L_3/Cu(NO_3)_2$, $L_3/Cu(CH_3CO_2)_2$ and $2L_4/Cu(CH_3CO_2)_2$ with the reaction rate respectively of 7.90, 7.27, 8.64 and 6.66 µmol.L^{-1}.min^{-1}). We notice also that copper acetate gives good oxidation rates with **L_2**, **L_3**, and **L_4**.

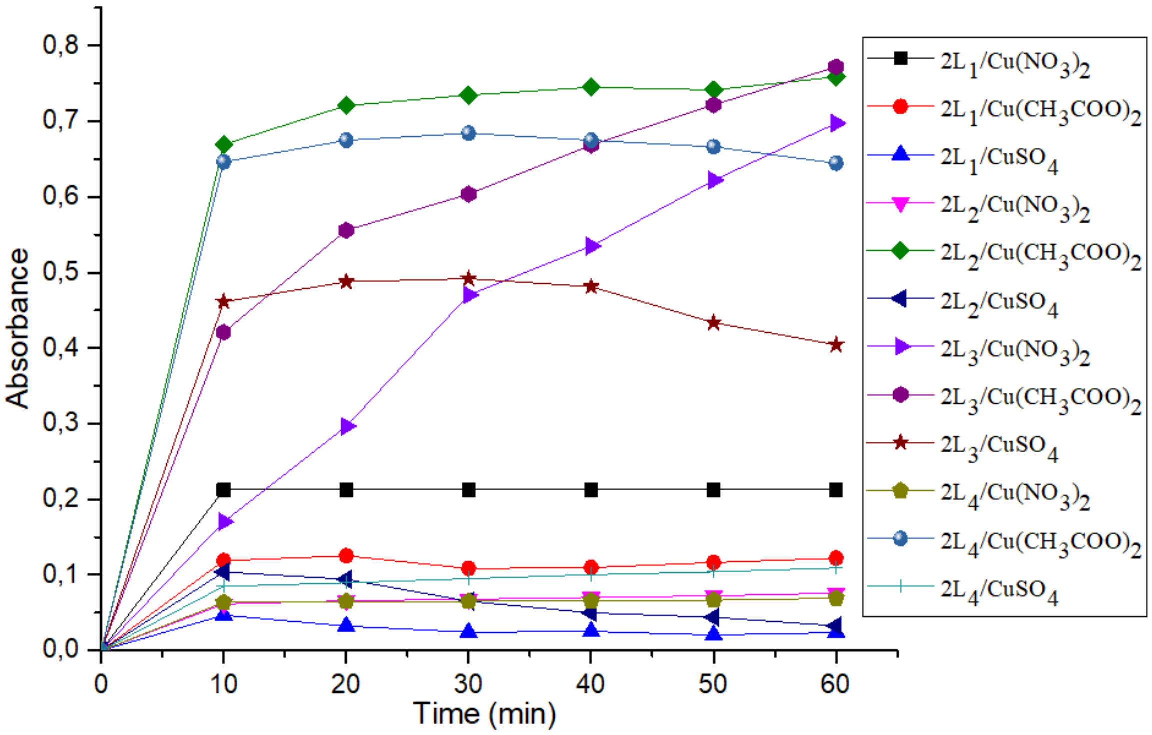

Figure 5. Oxidation of catechol in the presence of complex as formed by $L_1 – L_4$ and different metals salts (2L/1M) in methanol.

Table 2. Reaction rate V (µmol. L^{-1}.min^{-1}) of catechol oxidation in methanol (2Ligand/1Salt).

L/M	Cu(NO3)2	$Cu(CH_3CO_2)_2$	$CuSO_4$
L1	2.21	1.27	0.25
L2	0.79	7.90	0.34
L3	7.27	8.64	4.21
L4	0.72	6.66	1.14

3.1.3. Catalytic study of combinations (1L/2M) in MeOH

Figure 6 shows that the absorbance of o-quinone is greater when the catechol oxidation reaction is catalyzed by the complex formed by the ligand **L_3** and the copper (II) salt ($Cu(CH_3CO_2)_2$) and for the other two copper salts (II) a precipitate formed is observed leading to unstable absorbance values.

Catechol oxidation rates in the presence of combinations (1L/2M) based on ligands (L1-L4) and various copper salts (Ⅱ) in MeOH.

According to the results obtained in this **Table 3**, it is noted that all the copper complexes formed in-situ with the ligands **L_1-L_4** catalyze the oxidation reaction of catechol to o-quinone, and that the best catecholase activity was recorded with the complex formed from **L_3** and the metal salt $Cu(CH_3CO_2)_2$ (27.29 µmol.L^{-1}.min^{-1}).

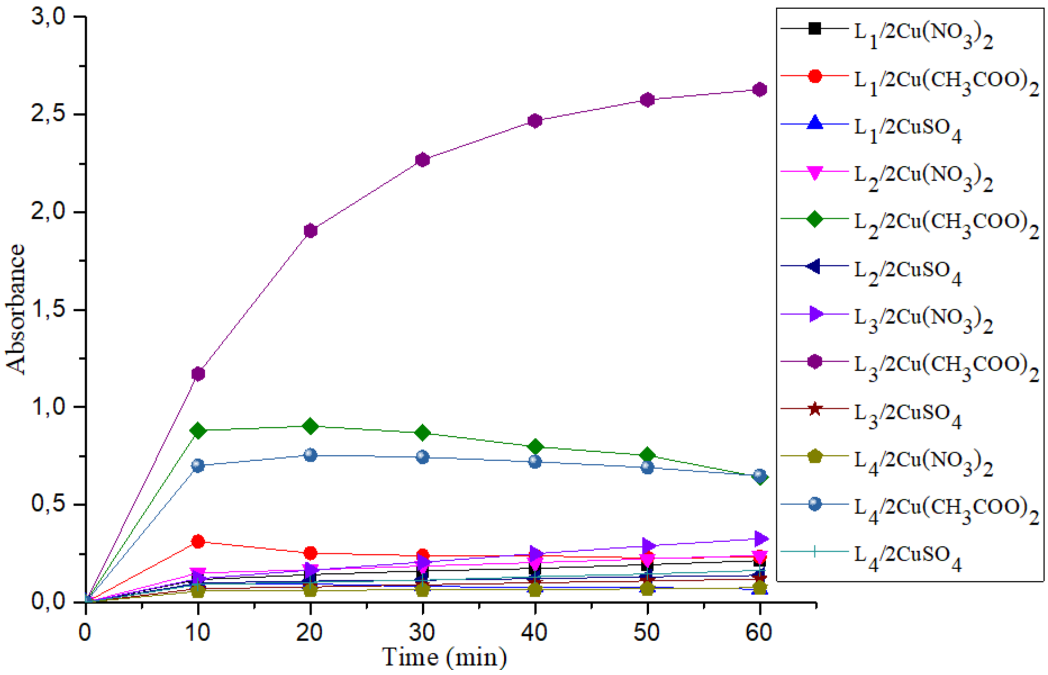

Figure 6. Oxidation of catechol in the presence of complexes formed by L_1-L_4 and different metals salts (IL/2M) in methanol.

Table 3. Reaction rate V (µmol. L^{-1}.min^{-1}) of catechol oxidation in methanol (1Ligand/2Salt).

L/M	$Cu(NO_3)_2$	$Cu(CH_3CO_2)_2$	$CuSO_4$
L1	2.23	2.43	0.70
L2	2.61	6.68	1.44
L3	3.39	27.29	1.24
L4	0.79	6.76	1.71

After comparing the results shown in **Tables 2** and **3**, it seems that there is a difference in oxidation rate values. This difference can be related to the effect of ligand concentration, which can be explained by the nature of the coordination environment. So, the effect of the nature of the counter anion on the catalytic activity was noted, and we notice that the best results were obtained with the CH_3COO^- ion. This allowed us to consider that the counter anion contributes to the coordination environment, and its nature strongly influences the catalytic activity of complexes.

3.2. Solvent Effect

To show the effect of the solvent on the catechol oxidation reaction, we carried out the same experiments under the same experimental conditions for the **L_2**, **L_3**, and **L_4** ligands, using as solvents: methanol (MeOH), tetrahydrofuran (THF), and N, N-Dimethylformamide (DMF). Study the different combinations based on **L_2**, **L_3**, and **L_4** ligands and copper salts (II) in MeOH, THF, and DMF. The results of **Table 4** show that the change in the absorbance of o-quinone is important (speed of oxidation of 11.92 µmol.L^{-1}.min^{-1}) when the THF solvent is used for the copper complex formed by the ligand L_4 and the metal salt $Cu(CH_3COO)_2$ with a speed of oxidation of 11.92 µmol.L^{-1}.min^{-1}. It is also observed that the different combinations in MeOH have good rates. On the other hand, the rate of absorbance remains average when using DMF as solvent (**Figures 7** and **8**).

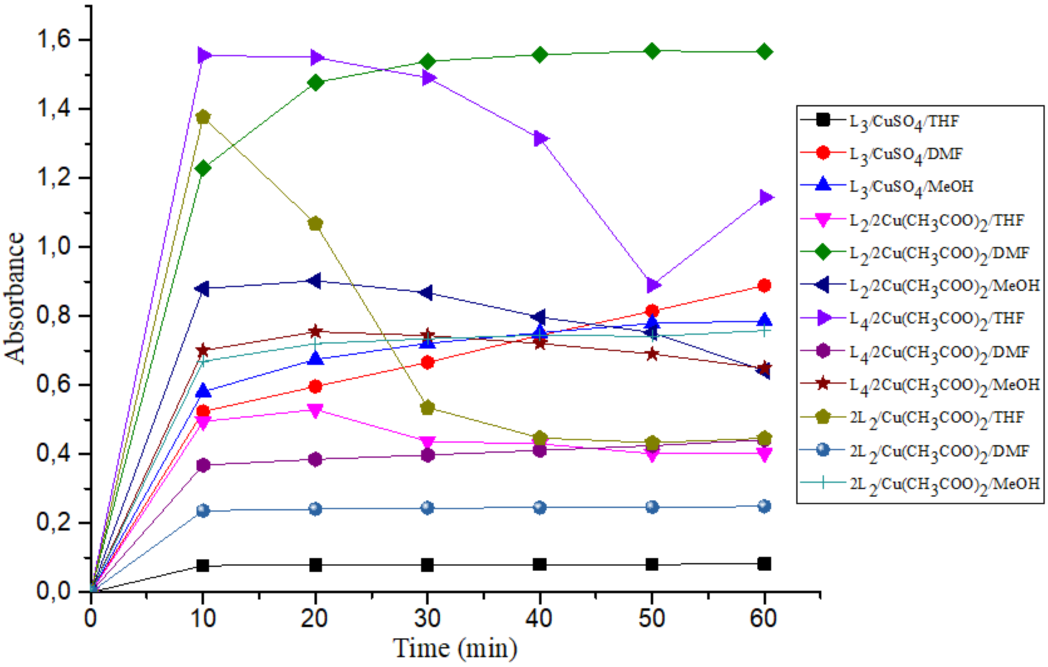

Figure 7. Oxidation of catechol in the presence of ligands with copper (II) salts in THF, DMF and MeOH.

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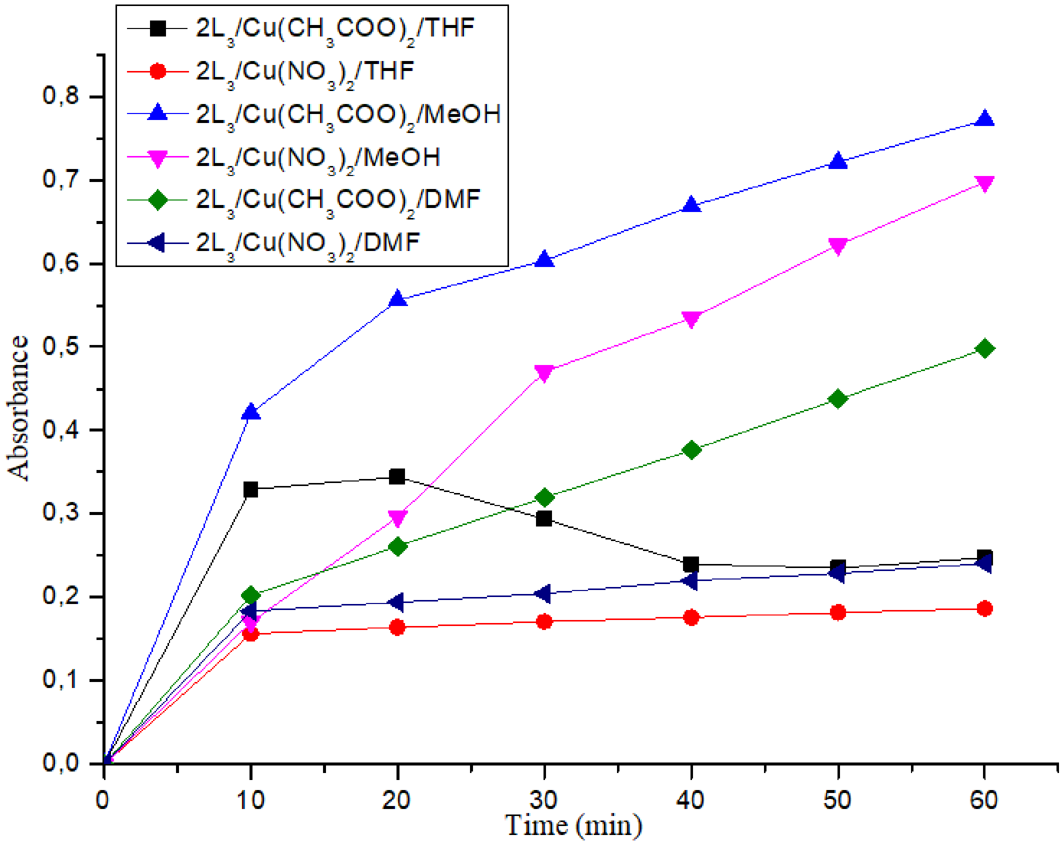

Figure 8. Oxidation of catechol in the presence of ligand L_3 with Cu $(CH_3COO)_2$, $Cu(NO_3)_2$, (2L/1M) in THF, DMF and MeOH.

Table 4. Catechol oxidation rates for combinations in three solvents in µmol.L^{-1}.min^{-1}.

Number of equivalences	Ligand	Copper(II) salt	Solvents	V_M (µmol.L^{-1}.min^{-1})
1L-2M	**L_4**	$Cu(CH_3CO_2)_2$	MeOH	6.76
			THF	11.92
			DMF	4.60
2L-1M	**L_3**	$Cu(CH_3CO_2)_2$	MeOH	8.64
			THF	2.57
			DMF	5.19
		$Cu(NO_3)_2$	MeOH	7.27
			THF	1.94
			DMF	2.50
2L-1M	**L_2**	$Cu(CH_3CO_2)_2$	MeOH	7.90
			THF	4.65
			DMF	2.58

3.3. UV-VIS Spectrophotometric Study

From the results obtained by UV-Vis spectrometry, it is found that the nature of the solvent used has a significant effect on the catalytic activity of the combinations studied. THF, which is a polar aprotic solvent, appears to be a better solvent than MeOH which is polar protic. If we consider that the physical parameters, generally known, of solvents like dielectric constant, dipole moment, polarity, etc, have no significant role in the change of the activity of the complexes towards the oxidation of catechol. It is the coordinating power or protic nature of solvents that play a key role in changing the catalytic activity of complexes. Polar protic solvents (such as methanol) can very strongly solvate X^- anions (with $X^- = CH_3COO^-$, SO_4^{2-}, NO_3^-) through hydrogen bonds. So these anions become isolated and thus are not very reactive in that sort of solvent. The cation (Cu^{2+}) is also solvated by the electronegative pole of the solvent, this solvation is less strong than with a hydrogen bond. The cation is moderately reactive and facilitates the formation of the complex and thus increases the rate of oxidation of the catechol. To validate the important catalytic activity of the combination (2L/1M) of L_4/Cu $(CH_3COO)_2$, $L_4/Cu(NO_3)_2$, and $CuSO_4$ in MeOH (the best catalysts of our complexes in the solvent MeOH). The kinetics of o-quinone formation and the change in absorbance was recorded every 5 min. Kinetic experiments were performed at room temperature (**Figures 9-12**).

The appearance of an intense band at 390 nm, explains that the four combinations are good catalysts for the oxidation of catechol to o-quinone. The band intensity is greater when using the $L_4/Cu(CH_3COO)_2$ combination in MeOH (**Figure 10**), which confirms that the $L_4/Cu(CH_3COO)_2$ combination is the best catalyst for our complexes.

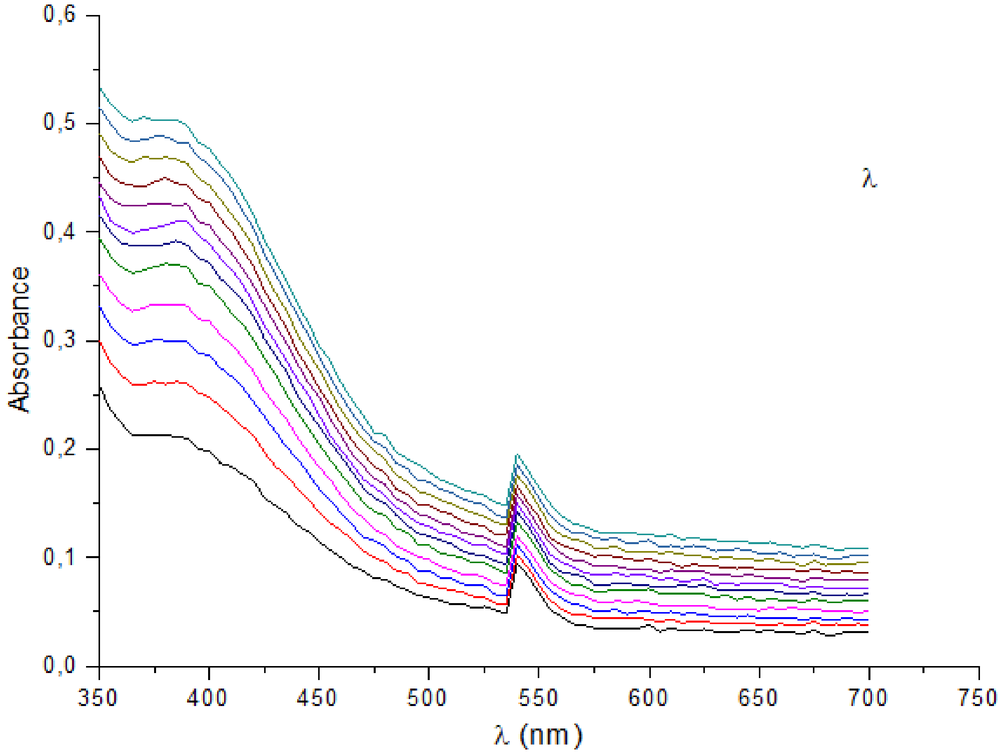

Figure 9. Absorbance spectrum of o-quinone as a function of time for the combination $L_3/CuSO_4$ in the MeOH.

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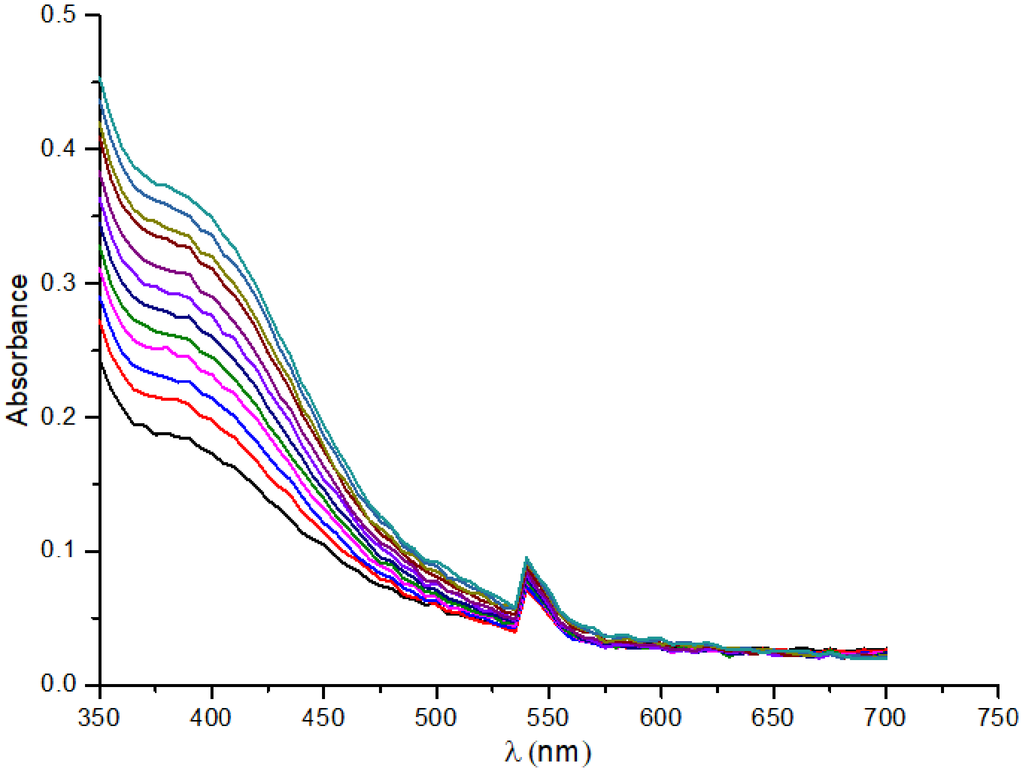

Figure 10. Absorbance spectrum of o-quinone as a function of time for the combination $2L_3/Cu(CH_3COO)_2$ in the MeOH.

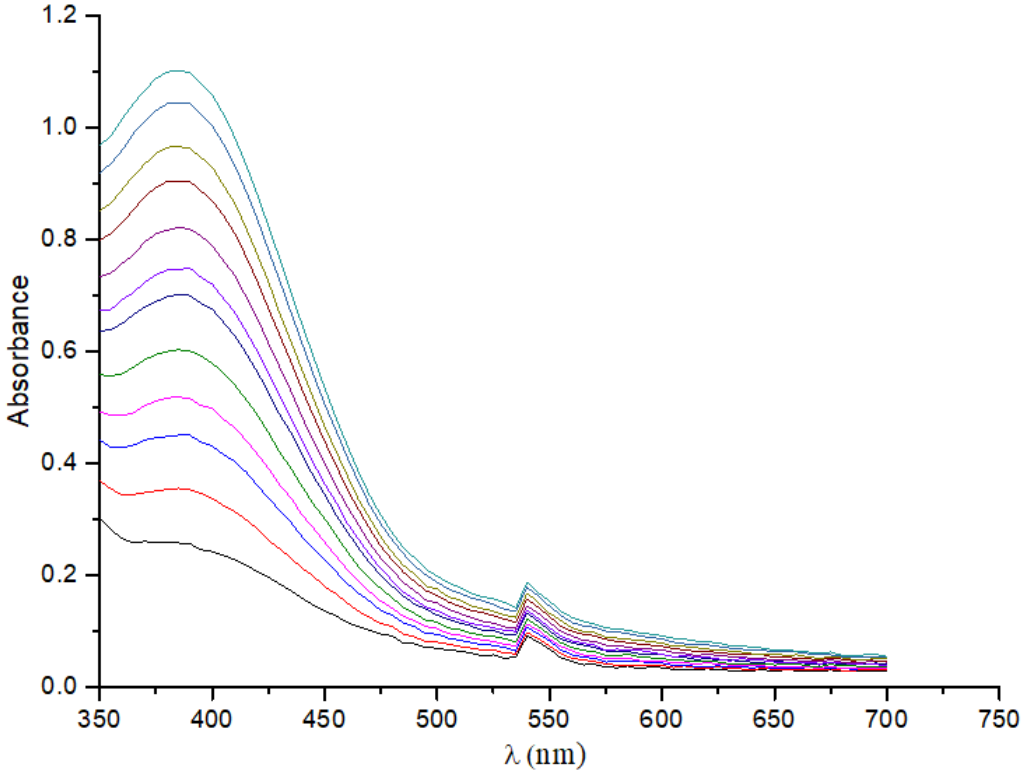

Figure 11. Absorbance spectrum of o-quinone as a function of time for the combination $L_4/2Cu(CH_3COO)_2$ in the MeOH.

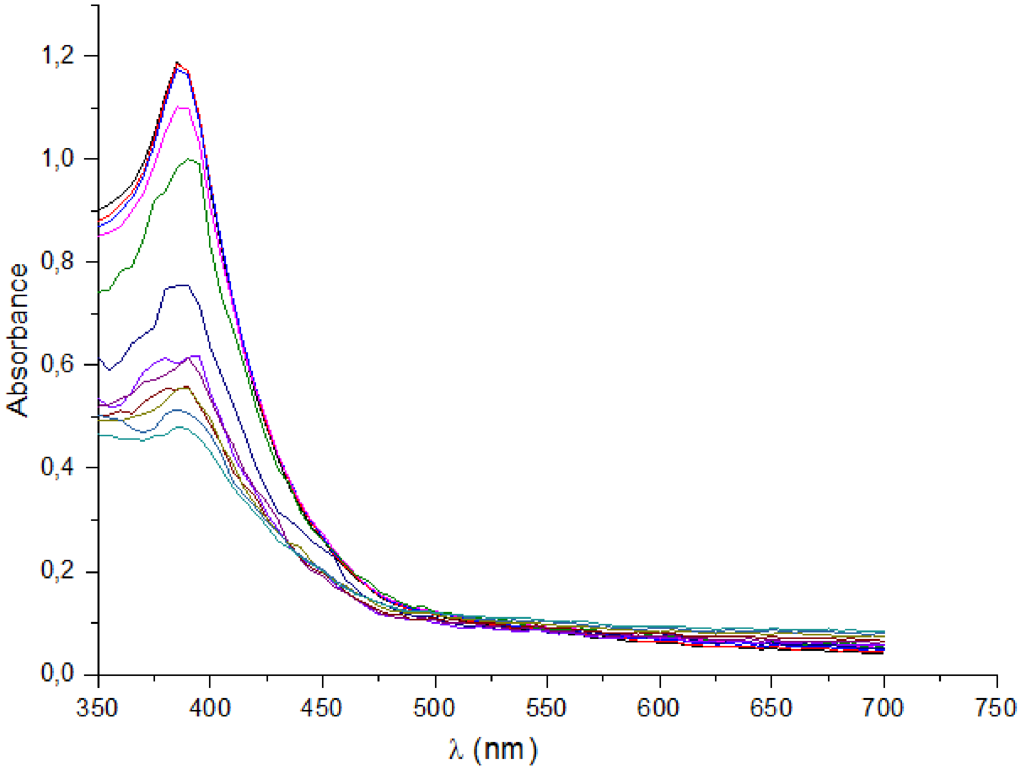

Figure 12. Absorbance spectrum of o-quinone as a function of time for the combination $L_2/2Cu(CH_3COO)_2$ in the MeOH.

3.4. Kinetic Study

A kinetic study was carried out by determining the constants *Km* and *Vmax* of the oxidation reaction of catechol. The method consists in plotting the graph representing the V_i as a function of the substrate concentration (catechol). We carried out this study by using a solution of the combinations $L_2/Cu(CH_3COO)_2$ (2L/1M, MeOH), $L_3/Cu(CH_3COO)_2$ (2L/1M, MeOH), $L_4/Cu(CH_3COO)_2$ (1L/2M, MeOH) and $L_4/Cu(CH_3COO)_2$ (1L/2M, THF) treated with different concentrations of catechol (from 10^{-3} mol.L^{-1} to 8.10^{-1} mol.L^{-1}) under ambient conditions. For the graphical determination of these two constants (*Km* and V_{max}), there are Michaelis-Menten graphical representations that present the results, allowing more precise extrapolations.

According to **Figure 13**, the kinetic study for the L_4 ligand shows that there is a linear relationship between the initial rates and the substrate concentration, therefore the Michaelis-Menten model is applied to obtain the kinetic parameters of the better catalyst. The *Vmax* speeds of $L_4/2Cu(CH_3COO)_2$ combinations in THF is 30.94 µmol.L^{-1}.min^{-1}, for $L_4/2Cu(CH_3COO)_2$ in MeOH is 69.38 µmol.L^{-1}.min^{-1}, for $2L_3/Cu(CH_3COO)_2$ is 48.66 µmol.L^{-1}.min^{-1} (**Table 5**), with a low value of *Km* for the $L_4/Cu(CH_3COO)_2$ combination, which explains that the affinity is strong in MeOH, which confirms that in our case the $L_4/Cu(CH_3COO)_2$ combination gives a better result for the oxidation of catechol.

As shown in **Table 5**, the value *Km* (0.019 mol.L-1) and maximal rate *Vmax* (69.38 µmol.L^{-1}.min^{-1}) for the four catalysts are presented and the best one is $L_4/2Cu(CH_3COO)_2$ in methanol for the catechol oxidation reaction.

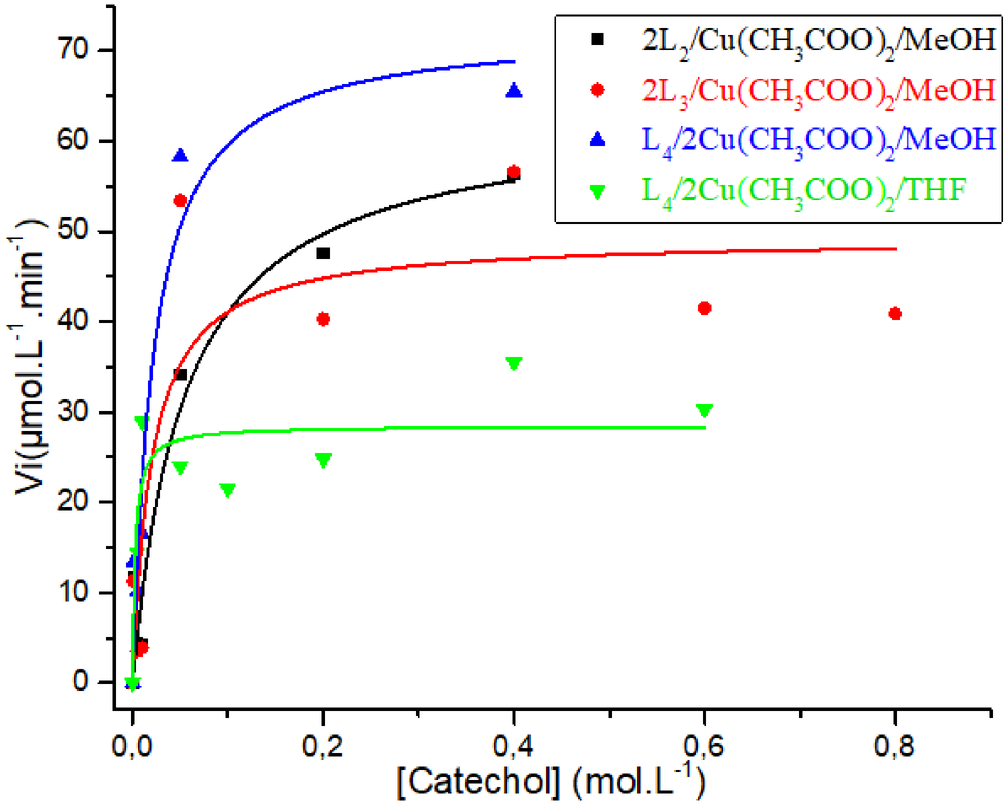

Figure 13. Dependence of the reaction rates on the catechol concentrations for the oxidation reaction catalysed by some complex formed in situ.

Table 5. Values of the *Vmax* and *Km* constants.

	Vmax **(µmol.L^{-1}.min^{-1})**	***Km*** **(mol.L^{-1})**
$2L_2/Cu(CH_3COO)_2$ (MeOH)	56.51	0.0430
$2L_3/Cu(CH_3COO)_2$ (MeOH)	48.66	0.0190
$L_4/2Cu(CH_3COO)_2$ (MeOH)	69.38	0.0190
$L_4/2Cu(CH_3COO)_2$ (THF)	*30.94*	*0.0019*

3.5. Proposed Reaction Pathway

The mechanism of the oxidation reaction of catechol to o-quinone is based on an earlier proposal (Solomon *et al*., 1996) and a recent series of crystal structures of various catechol oxidase intermediates (Klabunde *et al.*, 1998). The catechol molecules are cyclically oxidized and dioxygen is reduced to hydrogen peroxide (**Figure 14**).

The mechanism of catecholastic activity starts from the complex. catechol binds to the complex, followed by the reduction of copper (II) to copper (I), followed by oxidation of the substrate to the first o-quinone and formation of the oxidation of copper (I) to copper (II).

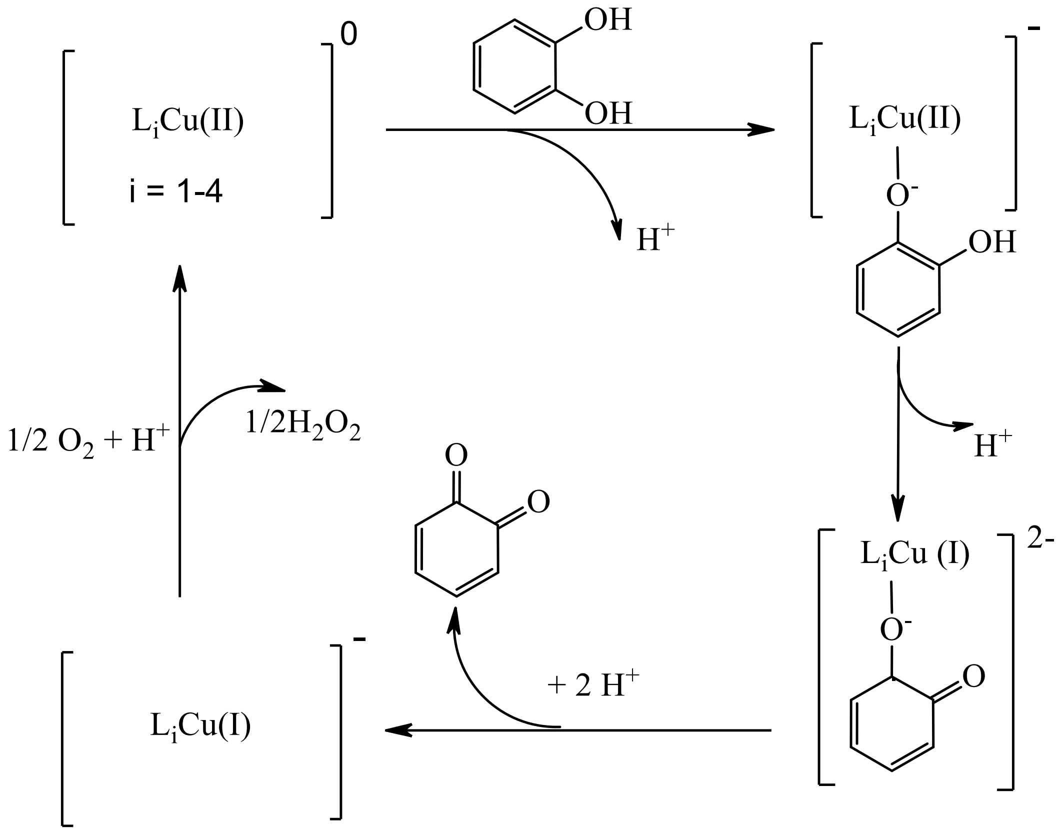

Figure 14. Proposed mechanistic pathway of the catalytic oxidation of catechol using complex formed by ligands L_i and Copper (II) salts.

4. CONCLUSION

In this work, we have studied the catalytic activity of heterocyclic ligands with different copper (II) salts. We have found that all combinations can catalyze the oxidation reaction of catechol to o-quinone but with different speeds at ambient conditions, using atmospheric oxygen as oxidant (catecholase activity). We have also examined the catecholase activity of ligands with different copper (II) salts. The combinations formed act as catalysts for catecholase activity at room temperature. We have found efficient catalysts for the catechol oxidation reaction, and we have also studied the kinetics of this reaction by the Michaelis-Menten model. The results are in agreement with this model. We have also shown that the catecholase activity of the combinations studied is influenced by several parameters such as the nature of the solvent, the nature of the ligand, the nature of the counter anion, the concentration of the ligand, and the concentration of the substrate.

5. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. The authors confirmed that the paper was free of plagiarism.

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