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Physicochemical Properties and Biological Activities of Novel Hydrazonate Copper Complexes

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Abstract

The objective of this research was to elucidate the biological effect of novel compounds derived from natural product of syringaldehyde through novel semi-synthetic method in order to investigate the physicochemical properties and biological activities by using DPPH and FRAP techniques and its antibacterial activities against *Klebsiella* spp., *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus*. Moreover, to examine its ability against breast cancer cell line (MCF-7). The results showed that the syringaldehyde hydrazonate copper complexes possessed the covalent bonds with square-planar structure. In terms of antioxidant DPPH activities, it was found that syringaldehyde hydrazone possessed high potency against DPPH free radicals, with respect to syringaldehyde hydrazonate copper complexes. On the other hand, all compounds possessed low reducing properties for changing Fe^{3+} to Fe^{2+} in FRAP technique. For antibacterial activities revealed that the ligand **L1** and **L5** possessed high effect on *Pseudomonas aeruginosa*, but for all copper complexes possessed high potent antibacterial susceptibility to four bacteria with concentration dependence. For anti-breast cancer cell line (MCF-7), it was found that all compounds possessed high potent anticancer susceptibility with low IC_{50}, especially, compound exhibit highly potency effective is **C5** (IC_{50} 9.75 μM). The tendency of anticancer effect from high to low was **C5** > **C2** > **C1** > **C4** > **C3**. Therefore, all synthetic compounds obtained from the present research possibly develop as the antibacterial drugs and the drugs for curing the diseases caused by free radicals, including breast cancer in metastatic phase. The most important feature of these drugs was the high specificity to the target and harmless to the normal cells.

Keywords

Schiff Base, Hydrazone, Natural Product, Anticancer, Antibacterial, Anti-Free

Radical

1. Introduction

Cancer causes from the mutation of genes that allow cells to divide unrestrictedly. Acquisition mutations can be caused by genetics (inherited) or other external factors. Acquired mutations are caused by conditions that are constantly triggered such as smoking or exposure to UV rays, and sometimes it can be caused by years of exposure to carcinogens [1]. When these risk factors are combined with other risk factors, many more, thus make it difficult to diagnose the real cause of cancers. But the most important thing is risk factors such as carcinogens, chemicals or dietary components, should be avoided [2], consumption of alcoholic beverages, and a diet high in fat and low in fiber, which is directly linked to the development of many cancers. But should consume a variety of vegetables and fruits, because it contains antioxidants and micronutrients necessary for health [3]. Normally, when the body is exposed to carcinogens, it doesn't happen immediately because DNA has a mechanism to repair it [4]. But if the body is exposed to carcinogens on a regular basis, it can result in mutations or changes in genes that cause cancer called oncogenes and also affect the tumor-suppressor genes such as p16, p53 and BRCA 1, which signal cells stop dividing. In the abnormal condition, tumor-suppressor genes are not stimulated to work but oncogene is, affecting the cell is grown and continuously developed [5]. This makes people die of cancer with the approximate number of 50,000 people per year. For the treatment of cancer today is still difficult to treat [6]. Even trying to develop a drug and modern tools but it can't be detected while the cancer cells are still small or in the early stages [7] [8]. Existing treatment methods, such as surgery, will work well. If detected in the early stages, there is also radiation and chemical use as well [9]. Depending on the diagnosis of a skilled physician. As for chemotherapy, it is considered one of the important treatments for this disease [10]. But the drugs that are currently used only provide relief from the symptoms. In addition, some drugs have many side effects because they lack specificity to cancer cells. And some types develop drug resistance when used to treat patients for a long time [11] [12]. In addition to the aforementioned cancer, there is another disease that is a global public health problem, such as a disease caused by bacteria [13], especially diseases caused by infection *Staphylococcus aureus* because it is currently resistant to methicillin [14]. Patients with this infection are difficult to treat. Therefore, the research team has tried to develop researching new, more effective substances are specific to destroying cancer cells that are not toxic to normal cells and it's not too expensive. Substances such as Schiff bases and Schiff bases conjugated with essential metal elements make the new compounds specific to the target cells. The bioactivity against bacteria of these compounds was also studied.

2. Materials and Methods

2.1. Chemical Reagents and Bacteria

All chemicals were purchased from Sigma-Aldrich, Fluka and Agar Powder, reagent grade and were used as received. For bacteria test strains both Gram-negative (*Klebsiella* spp. and *Pseudomonas aeruginosa*) and Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) were obtained from Department of Microbiology, Khon Kaen University.

2.2. Synthesis Hydrazone Ligands or Schiff Bases

All ligands were prepared by condensation of syringaldehyde with one of the five hydrazides indicated (Scheme 1) in the molar ratio 1:1 in MeOH (5 ml) with constant stirring at 40˚C for 6 hrs and concentrating the reaction mixture before allowing it to cool at room temperature. The products were obtained with high percentages of the yield as shown in Table 1.

2.3. Synthesis Copper Complexes

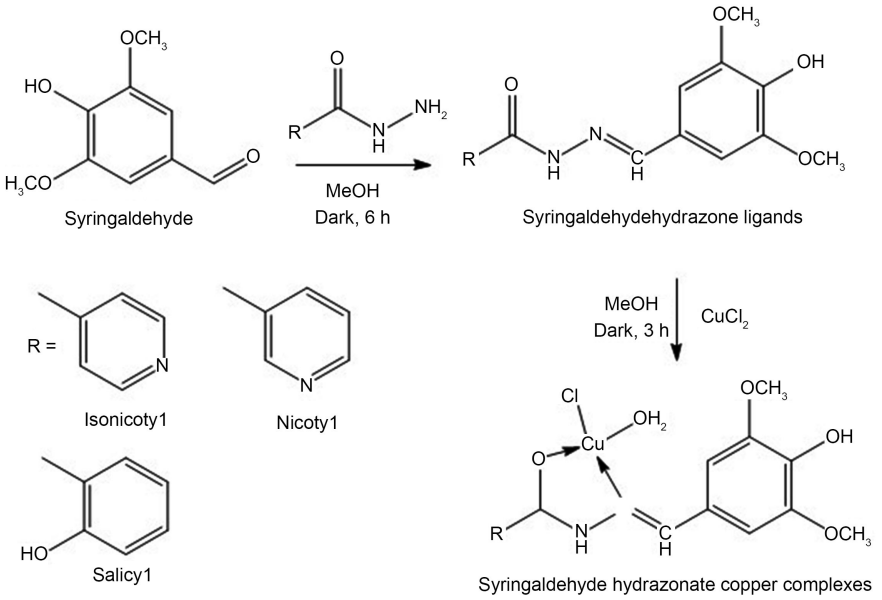
Copper (II) chloride dihydrate was added to the methanolic solution of syringaldehyde hydrazones in the molar ratio of 1:1 over a period of 3 hrs with constant stirring in dark. The products were separated by centrifugation and dried in vacuum.

2.4. Antioxidant Activity by DPPH Method

The assay was performed as described Sharma *et al.* [15], the DPPH radical scavenging activity of the compounds was measured. Briefly, 0.2 mM 2,2-Diphynyl-1-picrylhydrazyl (DPPH) solution was prepared with methanol. 100 µL sample was added to 3.9 cm^3 DPPH solution and was incubated in dark for 30 min at a temperature of 37˚C then the absorbance was measured at λ_{max} = 517 nm, calculate the percentage of anti-free radical activities.

Table 1. Physicochemical properties of samples.

Trivial name	Code	Percent yield	Melting point (˚C)	Color of samples	Solvents
1. SRA-Sc	L1	82	103.1	brown	MeOH, DMSO, DMF
2. SRA-Sc-Cu	C1	67	134.0	green	MeOH, DMSO, DMF
3. SRA-Tsc	L2	80	116.2	yellow	MeOH, DMSO, DMF
4. SRA-Tsc-Cu	C2	59	178.7	green	MeOH, DMSO, DMF
5. SRA-INH	L3	91	218.9	yellow	MeOH, DMSO, DMF
6. SRA-INH-Cu	C3	72	198.7	green	MeOH, DMSO, DMF
7. SRA-Nico	L4	89	218.8	white	MeOH, DMSO, DMF
8. SRA-Nico-Cu	C4	69	211.4	green	MeOH, DMSO, DMF
9. SRA-Sal	L5	87	233.4	brown	MeOH, DMSO, DMF
10. SRA-Sal-Cu	C5	65	288.9	green	MeOH, DMSO, DMF



Scheme 1. General synthetic scheme for preparation of syringaldehyde hydrazone ligands and their copper complexes.

2.5. Antioxidant Activity by FRAP Method

Prepare FRAP (ferric reducing/antioxidant power) by preparing acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridly-s-trizine) in solution 40 mM of HCL, and 20 mM of ferric chloride and then mix at the proportion of 10:1:1 (v/v). This will get FRAP solution, then prepare sample solution in purified water with the required concentrations [15].

2.6. Antibacterial Activities

All the newly synthesized compounds were screened for antibacterial activity against both gram-negative (*Pseudomonas aeruginosa* and *Klebsiella* spp.) and gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) by determining minimal inhitory concentration (MIC) and minimal bactericidal concentration (MBC) using the broth dilution method. Bacterial colonies were taken from fresh cultures and incubated in the tubes containing 5 mL of trypticase soy broth at 37˚C for 6 hours until achieving visible growth. Turbidity was adjusted to 0.5 McFarland standard by adding 0.05 mL of 1% w/v $BaCl_2{\cdot}2H_2O$ in phosphate buffered saline (PBS) to 9.95 mL of 1% v/v H_2SO_4 in PBS. Finally, 10 mg of each compound was dissolved in 10 mL of dimethyl sulfoxide (DMSO) to obtain concentration if 1 mg/mL [16].

2.7. Anticancer Activity to Breast Cancer Cell Line

By using MCF-7 cells during growth stage (9×10^4 cells/mL) 45 µL. Add sample element 5 µL diluted by DMSO 5% mixed with 384-well plated and incubate at a temperature of 37˚C with 5% of CO_2 for 3 days, then add solution resazurin 62.5 µg/mL 12.5 µL, incubate 4 hours at a temperature of 37˚C. Then draw a graph

for the relation between the concentration and the value of response of cells to find IC_{50} [17] [18] [19].

2.8. Statistic

All experiments were carried out in triplicates. Data obtained were analyzed by using one-way analysis of variance (ANOVA) and Pearson's correlation coefficient was performed. Significant differences between the means at 95% ($p < 0.05$) level were considered statistically significant. Data was recorded as mean ± standard deviation.

3. Results

3.1. Physicochemical Characterization of Samples

Physical chemistry feature of compound from synthesis, both ligands; syringaldehyde semicarbazone (L1), syringaldehyde thiosemicarbazone (L2), syringaldehyde isonicotinic acid hydrazone (L3), syringaldehyde nicotinic hydrazone (L4), syringaldehyde salicylic hydrazone (L5) and complex between ligands and copper (II) ion are C1, C2, C3, C4 and C5. The synthesized substances will have color, molecular formula, melting point, percentage of elemental substances, and percentage of products in different figures as in Table 1 and Table 2 respectively.

Table 2. Percent yield, molecular weight and elemental analysis of samples.

Code	MW	% C	% H	% N
L1	239.26	50.15* (49.67)#	5.43 (5.80)	17.55 (17.72)
C1	382.55	31.48 (31.37)	3.42 (3.40)	13.20 (13.33)
L2	255.32	46.69 (46.60)	5.09 (5.23)	16.45 (16.51)
C2	389.87	30.78 (30.65)	3.33 (3.30)	10.77 (10.70)
L3	301.32	59.74 (60.09)	4.98 (5.14)	13.94 (13.19)
C3	418.32	41.03 (41.29)	4.06 (3.77)	9.54 (9.55)
L4	301.32	59.74 (59.79)	4.98 (5.14)	13.24 (13.19)
C4	418.32	41.03 (40.88)	4.06 (3.77)	9.84 (9.85)
L5	316.33	60.69 (60.67)	5.06 (5.09)	8.85 (8.85)
C5	450.88	42.58 (42.50)	3.55 (3.50)	6.46 (6.21)

*calc., #found.

All synthesized substances are polar compounds. But polarity is predominant in ligands (**L1**-**L5**). These ligands are reacted with copper (II) ions to form complexes (**C1**-**C5**) with reduced polarity due to is a reduced charge. This is caused by the neutralization of negatively charged ligands with positively charged copper (II) ion.

The hydrazonate Schiff base ligands employed in the present work were synthesized in good yields (80% - 91%) by the condensation reaction of corresponding syringaldehyde with selected hydrazide in methanolic solvent 1:1 stoichiometry. They yield green colored copper complexes when conjugated with $CuCl_2{\cdot}2H_2O$. Their compositional analyses indicate a general molecular formula as [M(ligand)(H_2O)Cl] and absence of conductivity in DMSO solvent suggesting a nonelectrolyte nature for them.

3.2. IR Spectra

The significant peaks in ir spectra of parent compounds and their probable assignments useful for determining the coordination mode of the present ligands are summarized in **Table 3**.

The parent syringaldehyde compound (precursor) exhibits the carbonyl stretching frequency at 1671 cm^{-1} which is replaced after the condensation reaction by the imino stretching frequency at 1600 - 1699 cm^{-1} and additional stretches at 957 - 976 cm^{-1} due to hydrazinic N-N and C=O linkages respectively. The appearance of the ν (N-H) and ν (C=O) stretching vibrations indicate that in the solid state these ligands essentially exist in the keto form [20]. The broad band at 1600 - 1615 cm^{-1} arises out of the vibrations of the delocalized (>C=N-N=C<) linkage [21]. The complexes also display a new band in the far-infrared region at 355 - 330 cm^{-1} which can be assigned to stretching frequencies. Following metal complexation, the imino stretch is shifted to lower wave numbers whereas the hydrazinic N-N band shifts to higher wave numbers indicating participation of the azomethine group in copper complexation. The additional broad absorption observed in the visible 750 - 940 nm for copper complexes correspond to $^2B_{1g}$ $^2B_{2g}$ transition in the square planar environment in these compounds [22].

3.3. The Magnetic Susceptibility and Electron Spin Resonance (ESR) Data

When syringaldehyde hydrazonate copper complexes are calculated for ESR, it shows the results as in **Table 4**.

It well-known that x-band ESR spectra of copper (II) complexes can provide valuable information about the coordination environment around copper (II) ions. The spectra of four coordinate complexes in frozen DMSO glass show four resolved copper hyperfine lines which is indicative of monomeric copper (II) complexes having axial geometry. The calculations of ESR parameters yielding following relationship in the g values $g_{//} > g_{\perp} > g_e$ (2.0023) is consistent with a

$d_{x^2-y^2}$ ground state in the square-planar environment similar to many analogous compounds reported in literature [23]. For copper (II) complexes the $g_{//}$ is a moderately sensitive function for indicating covalency. For the covalent complexes $g_{//}$ is less than 2.3 and for ionic environments it is normally 2.3 or larger. Present compounds showing $g_{//}$ is between 2.25 - 2.29 obviously have a significant covalent character in their metal-ligand bonds in agreement with the observation of Kivelson and Neiman [24]. The degree of distortion f ($=g_{//}/A_{//}$) appears to be an empirical index of deviation from idealized geometry. Values of 110 - 120 cm^{-1} are typical for planar complexes, whereas the range 130 - 150 cm^{-1} is characteristic of slight to moderate distortion and 180 - 250 cm^{-1} indicate considerable distortion [25]. The present compounds found to be ranking from 142 - 177 cm^{-1} suggestive of slight to moderate distortion in geometry.

Table 3. IR Spectra of syringaldehyde hydrazones ligands (**L1-L5**) and their copper complexes (**C1-C5**).

Compound	ν(N-H)	ν(C=O)	ν(N-N)	ν(C=N)	ν(C=N-N=C)	ν(C-O)	Cu-O	Cu-N
precursor	3282	1671 (vs)	-	-	-	-	-	-
L1	3377	1699 (vs)	976(w)	1590(s)	-	-	-	-
C1	-	-	986(s)	1537 (s)	1610 (sh)	1371(s)	561	482
L2	3229	1645	957	1550	-	-	-	-
C2	-	-	986	1537	1600 (sh)			
L3	3225	1653	964	1587	-	-	-	-
C3	-	-	980	1509	1615 (sh)	1323 (s)	570	485
L4	3222	1636 (vs)	960 (w)	1586 (s)	-	-	-	-
C4	-	-	976	1553	1609 (sh)	1372 (s)	556	490
L5	3291	1642	963 (s)	1587	-	-	-	-
C5	-	-	974	1564	1612	1357	511	480

vs = very strong, s = strong, sh = shoulder, w = weak.

Table 4. X-band ESR parameters on copper complexes of syringaldehyde hydrazones (**C1-C5**).

Compounds	X-band ESR parameters				
	$g_{//}$	$g_{\perp}$	$A_{//}$ (G)		f (cm)
			mT	$\times 10^{-4}$ cm^{-1}	
C1	2.25	2.07	12.09	127	177
C2	2.25	2.07	11.55	121	177
C3	2.29	2.06	15.11	162	142
C4	2.28	2.06	15.12	161	142
C5	2.25	2.07	12.06	127	177

ESR data recorded at 77 K in DMSO solvent.

3.4. Antioxidant Activities

The ability of anti-free radical activities by using the technique DPPH technique illustrate the results by the percentage of free radical caused from the reaction made between the substance used in experiment and solution DPPH, calculating the spectral absorbance value at λ_{max} 517 nm, the wave length value that DPPH can highly absorb. It is found that the value of spectral absorbance is changed. Hence, the sample substance has anti-free radical effect by making reaction with solution DPPH [26]. The DPPH free radical receive electron or hydrogen free radical from the sample substance used in experiment, resulting consistency and changed to be independent atom which can be observed from the spectral absorbance at the λ_{max} 517 nm. The calculation of inhibitory concentration 50% is illustrated in Table 5

It is found that the novel Schiff base ligands have anti-free radical effect DPPH at a high percentage. All complexes showed anti-free radical DPPH and has concentration dependence. as the ligands but have lower proficiency than the ligands because of the bigger structure resulting the steric effect that makes it difficult to provide hydrogen free radical. For the results of anti-free radical activity by reducing Fe^{3+} to be Fe^{2+} (FRAP technique), it is revealed that both ligands and complexes can reduce Fe^{3+} [27] [28].

3.5. Antibacterial Activities

Prepare solution of samples in different concentrations and test antibacterial growth ability for the type *Escherichia coli*, *Klebsiella* spp., *Staphylococcus aureus* and *Bacillus cereus* for 24 hours. Then measure clear zone which find the results as in Table 6 for their copper complexes.

From Table 6, it is found that all 5 complexes have antibacterial effect for all tested strains, especially **C3** and **C5**, good antibacterial effects against *Staphylococcus aureus*. Moreover, it is also found that all complexes have effects on the dose dependence in nature.

Table 5. Value of IC_{50} of sample substances of anti-free radical DPPH.

Samples	IC_{50} (Inhibitory concentration 50%)	
	DPPH	FRAP
SRA	136.73^{a}	128.16^{a}
L1	43.31^{h}	69.93^{d}
C1	51.07^{g}	26.20^{h}
L2	50.03^{g}	69.99^{d}
C2	88.61^{b}	66.03^{e}
L3	75.93^{d}	90.16^{c}
C3	62.22^{f}	18.46^{j}
L4	29.92^{i}	65.16^{f}
C4	81.52^{c}	25.25^{i}
L5	81.18^{c}	95.21^{b}
C5	70.36^{e}	35.40
C.V. (%)	2.43	1.59

Table 6. The MIC and MBC of the synthesized Schiff bases against the tested bacteria.

Bacteria	MIC and MBC (ppm)	Compounds				
		C1	C2	C3	C4	C5
Klebsiella spp.	MIC	4.50 ± 0.71	5.50 ± 0.71	1.50 ± 0.71	7.00 ± 0.00	3.00 ± 0.71
	MBC	4.50 ± 0.71	5.80 ± 0.71	2.00 ± 0.00	7.50 ± 0.71	3.50 ± 0.00
Pseudomonas aeruginosa	MIC	7.00 ± 0.00	8.50 ± 0.71	1.50 ± 0.00	1.50 ± 0.71	1.25 ± 0.00
	MBC	8.50 ± 0.71	9.00 ± 0.00	2.50 ± 0.71	2.00 ± 0.00	1.50 ± 0.71
Bacillus cereus	MIC	9.00 ± 0.00	7.50 ± 0.00	3.00 ± 0.00	8.00 ± 0.00	5.50 ± 0.71
	MBC	9.50 ± 0.71	8.00 ± 0.00	3.50 ± 0.00	9.50 ± 0.71	5.50 ± 0.00
Staphylococcus aureus	MIC	5.00 ± 0.00	11.00 ± 0.00	2.00 ± 0.00	3.50 ± 0.71	2.50 ± 0.71
	MBC	5.50 ± 0.71	12.50 ± 0.71	2.50 ± 0.71	3.50 ± 0.71	3.50 ± 0.71

Note: Values are expressed as Mean ± S.D (n = 3).

3.6. Cytotoxicity to Normal and Breast Cancer Cells

The researcher used the complexes to test the effect of anti-breast cancer cell in metastasis stage (breast adenocarcinoma MCF-7 cell line). In addition, the researcher also studies whether the selected solution has poisonous effect to normal Vero cell line, as in Table 7.

From Table 7, it is revealed that the complex substances **C1**, **C2**, **C3** and **C4** are not poisonous to the normal cell except **C5** which has poisonous effect in high concentrations. The effect of anti-breast cancer growth MCF-7 cell line is found that all sub stances have anti-cancer cell growth at a good effect with the value of IC_{50}, low value, especially the **C5** (IC_{50} 9.75 μM). The efficiency of anti-cancer cell effect of sample substances ranking from much to less is as follows: **C5** > **C2** > **C1** > **C4** > **C3**.

4. Discussion

This research has synthesized and characterization of physicochemical properties, elucidation their effect as anti-free radical scavenging activities by using the DPPH and FRAP techniques, the screening of the *in vitro* antibacterial activity against two Gram-negative bacteria (*Klebsiella* spp. and *Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and anti-breast cancer MCF-7 cell line and also cytotoxicity condition to the normal Vero cell line on syringaldehyde hydrazone Schiff base ligands and their hydrazonate copper complexes. The results from the synthesis are found that both ligands and their complexes achieved at a high percentage and also found that the complexes are covalent and have a square-planar shape. Radical scavenging activity of the substances using the technique of DPPH is revealed that the syringaldehyde hydrazone Schiff base ligands have a very high ability on anti-free radical DPPH, but the syringaldehyde hydrazonate copper complexes have lower ability on anti-free radical than independent ligands. For anti-free

Table 7. Cytotoxicity of the syringaldehyde hydrazonate copper complexes to normal Vero cell and breast cancer cell line (MCF-7).

Complexes	cytotoxicity to Vero cell		cytotoxicity to MCF-7 cell line	
	activity	IC_{50} (μg/mL)	activity	IC_{50} (μg/mL)
C1	noncytotoxic	-	active	15.25
C2	noncytotoxic	-	active	12.46
C3	noncytotoxic	-	active	23.37
C4	noncytotoxic	-	active	20.24
C5	cytotoxic	49.11	active	9.75

radical activity by reducing Fe^{3+} to be Fe^{2+} (FRAP) is found that ligands and their complexes show low ability to reduce iron because molecule has difficulty in providing single electron transfer. Antioxidants have a differing capacity to stop the propagation of free radicals the influencing this both the structure of the antioxidant and the structure of the compounds to be oxidized [29]. For antibacterial activity are found that ligand **L1** can be antibacterial substances for the type *Pseudomonas aeruginosa* and ligand **L5** can be antibacterial substance for the type *Staphylococcus aureus* (MRSA) and *Bacillus cereus*. For the complexes, it is revealed that all complexes against bacteria with concentrations dependence in manner. The disease caused from these bacteria is difficult to cure, especially caused from bacteria *Staphylococcus aureus* because these bacteria resist drug action to Methicillin taken nowadays. The results of this study are revealed that the **L5** can be antibacterial growth. It is therefore a new interesting discovery and also a new hope for patients caused from bacteria *Staphylococcus aureus* [30]. The ability on anti-breast cancer MCF-7 and cytotoxicity condition to normal cell are found that nearly all chemical compound is not poisonous to normal cell except **C5** which is poisonous at a high concentration. The effect against breast cancer growth MCF-7 cell line (human metastatic breast cancer) is found that all chemical compounds show significant inhibition against hormone dependent breast cancer cell line with IC_{50} < 25 μM, especially the **C5** (IC_{50} 9.75 μg/mL) as summarized in Table 7. Lending support to our earlier observation that copper is a key metal which helps in enhancing the biological activity [31] [32].

5. Conclusion

The synthesis of both novel ligands and their metal-organic compounds showed achieving a high percentage of productivity. For complexes were covalent and had a flat rectangular shape (square planar). The higher DPPH radical scavenging activity was found in syringaldehyde hydrazone Schiff base ligands than syringaldehyde hydrazonate copper complexes. Regarding the antioxidant effect by reducing Fe^{3+} to Fe^{2+} (FRAP) of all compounds, it was found the ligands and complexes were less able to reduce iron. Antibacterial activities are clearly indicated that copper conjugation synergistically enhances the antibacterial activities

of syringaldehyde hydrazone. Almost all compounds were non-toxic to normal cells except C5, which was toxic at high concentration. As for the anti-growth activity of human metastatic breast cancer cells (MCF-7 cell line), all compounds were effective against the growth of cancer cells.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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