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Study anticancer and antioxidant activity of the prepared compound 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2yl)hydrazinylidene] methyl}phenol

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Abstract:
The current study paid attention to the preparation of a chemical compound, 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol 2yl)hydrazinyli dene]methyl}phenol and its complexes. This ligand was used as an anti-cancer agent, as it gave high efficacy when tested on normal cells and cancer cells. This compound was also used as an antioxidant when compared with vitamin C. It was found to have high efficacy at low concentrations. This study included three axes, the first axis synthesis of chemical compound (2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol 2yl)hydrazinylidene]methyl}phenol)and its complexes with Ni(II), Cu(II), Fe(III), Cr(III) ions and characterization by mass spectra, 1HNMR and FTIR. The hyper-chem study of transition metal complexes suggests octahedral geometry for Fe^{+3} and Cr^{+3} ion, square planer geometry for Ni^{+2}, and Cu^{+2}, suggesting tetrahedral geometry. In the second step, the ligand was tested as an anticancer activity also, In the third step the ligand was tested as an antioxidant activity the prepared ligand showed good anticancer, and antioxidant activity

Key words: Thiadiazol, antioxidant, anticancer, heterocyclic, complex

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INTRODUCTION

Cancer is a fatal illness that nevertheless poses a serious threat to world health. After heart illnesses, it is the disease that occurs most frequently. Thus, one of the most strongly held objectives of modern medicinal chemistry is the creation of potent and effective novel antineoplastic medicines [1]. Breast cancer is the most common type of cancer in women. Although most breast cancers are benign and treatable with surgery, one-quarter have a latent and insidious nature, developing slowly but metastasizing quickly. Current medicines considerably slow tumor development, but recurrence is unavoidable, resulting in high fatality rates. Breast cancer cell behavior is seeded in its inception. Embryonic mammary cells have motile and invasive capabilities, and mammary development is characterized by cell mobility and alterations in cell contact.

Thiadiazols are heterocyclic compounds with a five-member ring, nitrogen, and sulfur [1]. As shown in Figure 1, they exist in nature in four isomeric forms: 1,2,3-thiadiazole, 1,2,5-thiadiazole, 1,2,4-thiadiazole, and 1,3,4-thiadiazole. Among heterocyclic compounds, 1,3,4-thiadiazole has emerged as a key building block for the creation of novel medications. Antibacterial, anti-inflammatory ,anticonvulsant, antituberculosis, antihypertensive , antioxidant , antiviral , anticancer , carbonic anhydrase inhibitors , and acetylcholinesterase inhibitory activities are among the biological activities of compounds containing the 1,3,4-thiadiazole scaffold. Furthermore, herbicides, fungicides, pesticides, insecticides, and bactericides are widely employed in agricultural applications [3-17]. The inclusion of the =N-C-S moiety may contribute to the biological activity of 1,3,4-thiadiazole moieties [18].

Oxidative stress is defined as an imbalance between the systemic expression of reactive oxygen species and the ability of a biological system to readily detoxify the reactive intermediates or repair the consequent damage [14]. The role of oxidative stress in neurodegenerative disorders such as Lou Gehrig's disease, Parkinson's disease, Alzheimer's disease, Huntington's disease, and Multiple Sclerosis is suspected [15]. Monitoring biomarkers such as reactive oxygen species, reactive nitrogen species generation, and antioxidants provides indirect evidence.

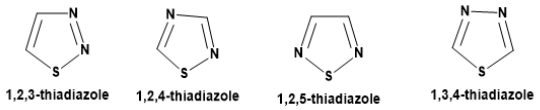

Fig 1. Thiadiazols

EXPERIMENTAL

Chemicals and instruments

All reagents and anhydrous solvents were used exactly as supplied by commercial vendors (Sigma-Aldrich, BDH, England, and Fluka). As chloride, all metal salts were employed. Melting points were calculated using the capillary technique on an Electro thermal IA9000 in Essex, UK, and are uncorrected. TLC was performed on

silica gel (60) F254 Merck, FT-IR spectra were recorded on a KBr disk using a Shimadzu model (Kyoto, Japan) spectrophotometer, and CHN microanalysis was performed using a Euro EA3000 elemental analyzer (Carlo Erba, Milan, Italy). 1H-NMR and 13C-NMR spectra were obtained using tetramethylsilane (TMS) as an internal standard on an Inova model Ultra shield 500MHz. The chemical shift was recorded as (=ppm), and the solvent was DMSO-d6.

The synthesis of ligand: The ligand (L) was synthesized by a multistep synthetic strategy which has been outlined

Synthesis of 2,5-Dimercapto 1,3,4-thaidiazole: For 25 hours, hydrazine hydrate (0.1 mol, 4.85 ml), carbon disulfide (0.2 mol, 12.6 ml), and KOH (0.2 mol, 11.2g) was refluxed. TLC was used to monitor the response. The surplus solvent was then distilled away, and the resultant solid was separated using 10% hydrochloric acid. After filtering the mixture, a dark yellow solid was recrystallized from ethanol. m.p = (162-164) C, compound yield = 78% . shown in Figure 2.

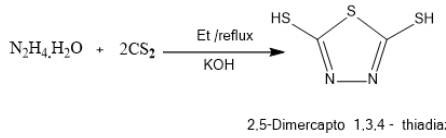

Fig 2. 2,5-Dimercapto 1,3,4-thaidiazole

Synthesis of 5-hydrazineyl-1,3,4-thaidiazole-2-thiole: For 10 hours, a combination of compound (1) (0.1 mol, 15g) in 50 ml absolute ethanol and (80%) hydrazine hydrate (0.1 mol, 5 ml) was refluxed. TLC was used to monitor the response. After allowing it cool to room temperature, pour in (100 ml) of ice water. The yellow solid product was filtered, washed with water, and recrystallized from ethanol, yielding 62% of the compound (2) as shown in Figure 3.

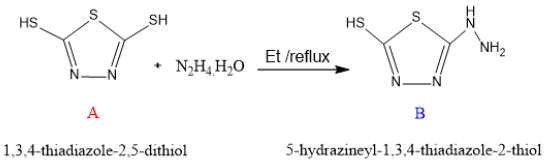

Fig 3. 5-hydrazineyl-1,3,4-thaidiazole-2- thiole

Synthesis of 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2-yl)hydrazinylidene] methyl} phenol: A mixture of compound (2) (0.02 mol, 2.96) in (20 ml) of absolute ethanol with 2- hydroxy-3-methoxy benzaldehyde (0.02 mol, 3.04g) add a few drops of glacial acetic acid was refluxed for (3 hrs.) The reaction was followed by TLC. Then cooled to room temperature. The dark yellow solid result was filtered off, washed with water, and recrystallized from ethanol. m.p: (d>170) °C, yield 85%. The sequence of steps is illustrated shown in Figure 4.

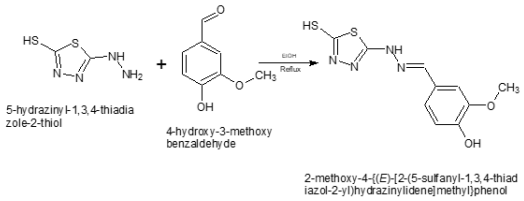

Fig 4. 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2-yl)hydrazinylidene]methyl} phenol

Preparation of complexes

The complexes were synthesized by mixing (0.001mol) from ligand with (0.001mol) of the different salts ($CrCl_3.6H_2O$, $FeCl_3.6H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, and $CuCl_2.2H_2O$) both alone in (15ml) ethanol absolute and refluxed for 2 hours. (monitored by TLC). then the precipitate was filtered and washed several times with ethanol or aqueous ethanol to remove unreacted salts or ligands, then the precipitated complexes were dried.

Anti-cancer activity

Cell Lines:

OLN-93 cell line a new permanent oligodendroglia cell line derived from primary rat brain glial cultures. This cell line obtained from Center of Biotechnological Research. No. of passage: 15.

Solutions and Media Used in Tissue Culture Technique:

Solutions and media used for cell culture were prepared according to Freshney, (2010) [19].

Solutions:

A-antibiotic solution

1. Streptomycin (1g/vial): It was prepared by dissolving vial contents in 5 ml of sterile distilled water to prepare a stock solution (200,000µg/ml). The stock was stored at -18°C. And 0.5 ml of it was added to 1 litter of culture media.
2. Benzyl Penicillin: It was prepared by dissolving the contents of one vial which has 106 IU in 5ml of sterile distilled water to prepare a stock solution (200,000 IU/ml). The stock was stored at -18°C. And 1 ml of it was added to 1 litter of culture media.

Sodium Bicarbonate Solution

The solution was prepared by dissolving 2.2 g of $NaHCO_3$ in 1000ml distilled water. The solution was sterilized by autoclaving and kept at 4°C until use [20].

Phosphate Buffer Saline (PBS)

This buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15 g NaH_2PO_4 and 0.2g Na_2HPO_4 in 900ml of distilled water, pH was adjusted to 7.2. The solution was sterilized by autoclaving and stored at 4°C until use.

Trypsin Solution

It was prepared by dissolving 1 g of trypsin powder in 100ml PBS and sterilized by filtration using Millipore's filter (0.22µm). The solution was dispensed into 10ml aliquots and stored at -20°C.

EDTA Solution

It was prepared by dissolving 1 g of ethylene-diamine-tetra acetic acid (EDTA) in 100ml of PBS and sterilized in autoclave for 10 minutes. The solution was dispensed in 10 ml aliquots and stored at 4°C.

Trypsin-EDTA Solution

It was prepared by mixing 20ml of trypsin solution, 10 ml EDTA solution and 370 ml PBS. The mixture was stored at 4°C.

Media:

Roswell Park Memorial Institute – 1640 Medium (RPMI)

A ready to use package (100 ml) RPMI was used throughout this study. The medium was already supplied with 4-(2-hydroxyethyl)-1piperazine-ethane sulfonic acid (HEPES) and L-glutamine as illustrated by manufacturer.

The medium was completed by adding the following ingredients:

Penicillin G	103 IU
Streptomycin	0.001 g
Sodium Bicarbonate	1%
Fetal Bovine Serum	10 %

Serum Free Medium

Serum free medium is RPMI-1460 excluded from fetal calf serum.

Methods:

Sterilization Methods

Moist Heat Sterilization

1. Solutions and some laboratory utensils were sterilized by autoclaving at 121°C, 15 psi for 15 minutes.
2. Dry Heat Sterilization: Electric oven was used to sterilize the glassware and others by heating at 180°C for 2 hours.
3. Filtration (Membrane Sterilization): Solutions sensitive to heat were sterilized by filtration using millipore's 0.22 µm in-diameter filters.

The Cytotoxic Effect of Cuprizone Compounds on Tumor Cell Lines
In the presence of different concentrations of levetiracetam.

Cell Line Maintenance

When the cells in the vessel formed confluent monolayer, the following protocol was performed:

1. The growth medium was aspirated and the cell sheet washed with PBS.
2. Two to three ml trypsin/EDTA solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubation at 37°C for 1 to 2 minutes, until the cells were detached from the vessel.
3. Fresh complete RPMI medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.
4. Cells were redistributed at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37°C in 5% CO2 incubator.

Cell concentration was achieved by counting the cells using the haemocytometer and applying the formula:

Total Cell Count/ml: cell count x dilution factor (sample volume) x 104

MTT Protocol:

The cytotoxic effect of Cuprizone 50mM with presence different concentrations from levetircatam was performed by using MTT ready to use kit (Intron Biotech):
Kit contents:

- MTT solution 1 ml x 10 vials.
- Solubilization solution 50 ml x 2 bottle.

Protocol

- Tumor cells (1x104 – 1x106 cells/ml) were grown in 96 flat well micro-titer plates, in a final volume of

200µl complete culture medium per each well. The microplate was covered by sterilized parafilm and shacked gently.

- The plates were incubated at 37°C, 5% CO2 for 24 hrs.
- After incubation, the medium was removed and two-fold serial dilutions of the Levetiracetam (200, 100, 50, 10, 2.5, and 0.5 µM/ml) were added to the wells.
- Triplicates were used per concentration as well as the controls (cells treated with serum-free medium). Plates were incubated at 37°C, 5% CO2 for selected exposure time (4 hrs).
- 50µM/ml of cuprizone was add to each well for 24 H.
- After exposure, 10 µl of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO2 for 4 hrs.
- The media were carefully removed and 100µl of solubilization solution was added per each well for 5 min.
- The absorbance was determined by using an ELISA reader at a wavelength of 575 nm. The data of optical density was subjected to statistical analysis in order to calculate the concentration of compounds required to cause 50% reduction in cell viability for each cell line, through the following equation:

$$Y = \frac{D + A - D}{1 + 10^{(x - logC)B}}$$

Statistical Analysis:

A one-way analysis of variance ANOVA (Duncan) was performed to test whether group variance was significant or not, statistical significance was defined as $p \leq 0.05$. Data were expressed as mean± standard deviation and statistical significances were carried out using Graph Pad Prism version 9.4(Graph Pad Software Inc., La Jolla, CA).

Antioxidant activity

- Preparation of solutions used in the antioxidant test: The following solutions were prepared according to Rajesh and Natvar.
- Methanol-DMSO Mixture (9:1 v/v) Solution: Methanol: DMSO mixture (9:1 v/v) solution was prepared by adding 9 volumes of methanol to 1 volume of DMSO.
- DPPH Radical Solution: DPPH radical was dissolved in DMSO: Methanol of (1:9) (v/v) mixture to prepare (0.1mg/ml) DPPH radical stock solution.
- Vitamin-C Solution: Ascorbic acid powder was dissolved in DMSO: Methanol of (1:9) (v/v) mixture to prepare a concentration of (0.1mg/ml) vitamin C stock solution.

Procedure

Antioxidant activity of some synthetic compounds (L_1) was detected by using DPPH radical scavenging assay according to the procedure described by Rajesh and Natvar as follows:

1. Methanol (130 µl) was added to each microtiter plate well.
2. Each sample (20 µl) (synthetic compounds, and vitamin C) was added separately.
3. Serial 10-fold dilution was done for each sample.
4. 50 µl of DPPH radical solution obtained in (3.1.4.3.1) was added for each well.
5. Microtiter plate was incubated at 37 0C for one hour with dark
6. Radical scavenging activity of samples against the stable DPPH radical was determined spectrophotometrically. The colorimetric changes (from deep- violet to light- yellow) when DPPH is reduced were measured at 517 nm.

RESULTS AND DISCUSSION

Analysis and physical measurements

Physical properties, molar conductance, and magnetic susceptibility of the ligand and its complex are shown in (Table 1).

Tab. 1. Physical properties

Yield %	M.P. ^{0}C	Color	M.Wt	Formula	No.
74	200-202	yellow	282	L4($C_{10}H_{10}S_2O_2N_4$)	1
69	212-215	Brown e	723	$C_{20}H_{20}Cl_3CrN_8O_4S_2$	2
71	218-220	yellow	416	$C_{10}H_{10}Cl_2CuS_2N_4O_2$	3
80	207-209	white	444	$C_{10}H_{10}Cl_3FeN_4O_2S_2$	4
76	217-219	Brown e	412	$C_{10}H_{10}Cl_2NiS_2N_4O_2$	5

FT-IR spectra

FT-IR spectroscopy is one of the important tools which used characterization of functional groups in the prepared ligand and was carried out using a KBr disc. The free ligand (L) exhibited nine major bands at (3464), (3194), (3094),(2994),(1619), (1603),(1574), (1399), (1359) and (1082) cm^{-1}. Which are corresponding with

(υO-H), (υN-H), (υC-H aro), (υC-H Elaf) (υC=N) oxo, (υC=C), (υC=N) endo, (υC-N-C) sym, (υ C-N-C) asy structure movement bands respectively, as shown in (Table 2) and (Figure 1-5). New bands were formed corresponding with the coordinated (M- N) and (M-O) bonds and shown at the region (628-650) cm^{-1}, (500) cm^{-1}respectively.

Tab. 2. FT-IR Spectra

Compound	L_1	Ni	Fe	Cr	Cu
OH υ	3464	3319	3206	3247	-----
NH υ	3194	3177	3206	3144	-----
Ar(C-H) υ	3094	3092	-----	3037	3094
Elf(C-H) υ	2994	2929	2935	2952	2993
(C=N) Azo υ	1619	1639	1621	1583	1619
(C=C) υ	1603	1613	1537	1518	1603
(C=N) Het υ	1574	1580	1495	1476	1584
Asy(C-O-C)	1399	1384	1377	1395	1397
Sym(C-O-C)	1359	1309	1339	1363	1360
Structural movement	1082	1077	1048	1075	1078
M-N υ	-----	633	650	632	628
M-O υ	-----	-----	500	-----	-----

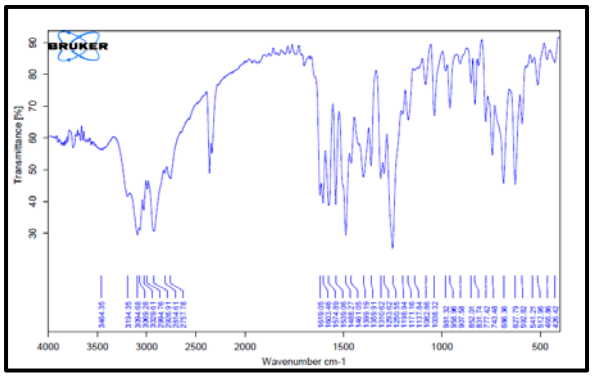

Fig. 1. FT-IR Spectra of The Ligand

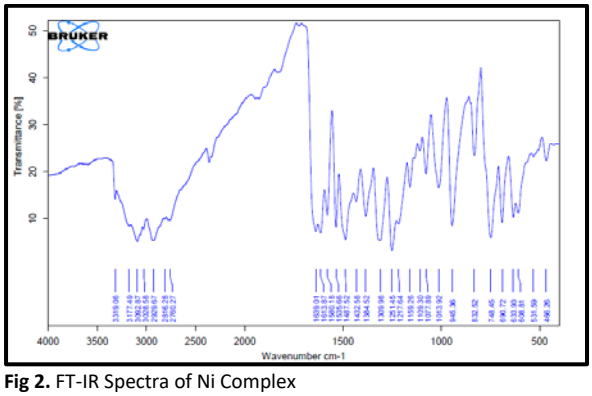

Fig 2. FT-IR Spectra of Ni Complex

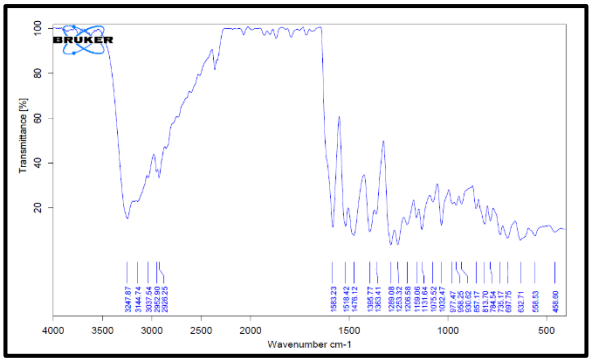

Fig 3. FT-IR Spectra of Cr Complex

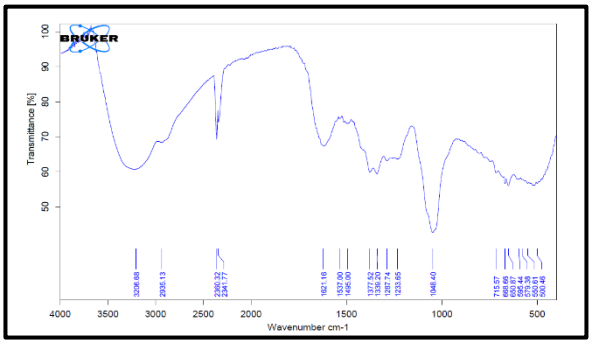

Fig 4. FT-IR Spectra of Fe Complex

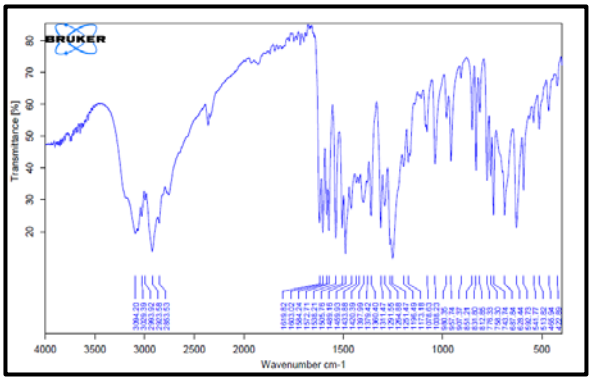

Fig 5. FT-IR Spectra of Cu Complex

Nuclear Magnetic Resonance Spectra (1H-NMR)

The 1HNMR spectra data of the 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2-yl)hydrazinylidene]methyl}phenol, was distinguished by the appearance of multiple peaks at (2.50 and 3.37 ppm) the first due to protons of the solvent (DMSO) and the second for the HDO, (3.84 ppm, 3 H) due to protons of methoxy group, (14.05 ppm , 2H) due to N-H(Exo) , N-H(endo) , proton, (6.96 -7.44 ppm, m, 3H) due to protons of aromatic rings,(9.77 ppm, 1 H) due to proton of azo methane group (-N=CH-), (10.30 ppm, s,1H) due to OH group, as shown in (Figure 6).

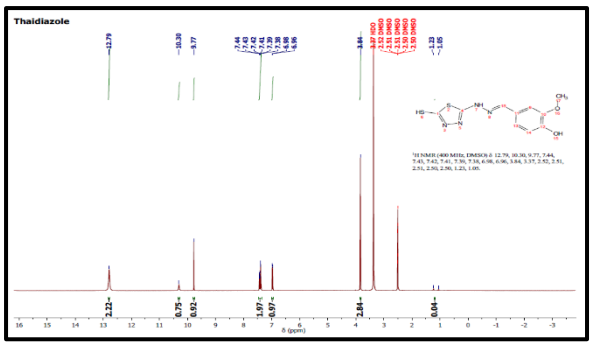

Fig. 6. 1HNMR Spectra of the ligand

Mass spectra

Mass spectra of the prepared ligand and its transition metal complex were recorded at a temperature of the room, the mass spectra of the ligand showed a molecular ion peak at 282 m/z which is by the molecular formula $[C_{10}H_{10}S_2O_2N_4]^{+\cdot}$, other peaks are due to the subsequent fragments such as (255 m/z,115 m/z,93 m/,132 m/z,150 m/z, 85m/z (respectively$[C_9H_7N_4OS_2]$ + $[C_2H_3N_4S]^+$, $[C_6H_5O]$+,$[C_2H_3N_3S_2]$ + $[C_8H_8NO_2]^+$,$[C_2HN_2S]^{+9}$ (Figure 7-11) [21-25].

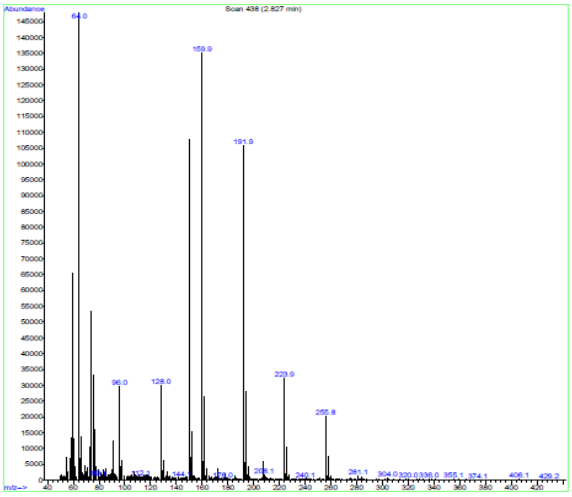

Fig. 7. MS Spectra of the ligand

The MS of the complex [Ni (L) Cl_2] shows a molecular ion peak [M0] at 412 m/z that is equivalent to the molecular mass of the complex. The other peaks are shown as follows:

- [Ni (L) Cl] $^+$ = 367 m/z
- [Ni (L)] $^+$ = 341 m/z

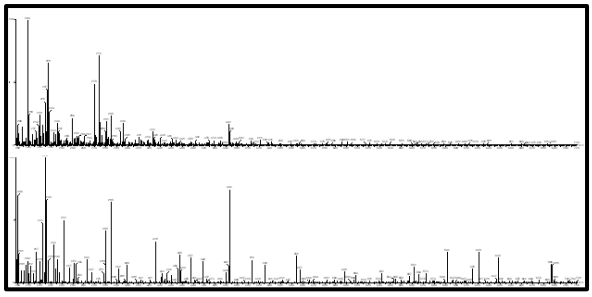

Fig. 8. MS Spectra of the Cu Complex

The MS of the complex [Cu(L) Cl_2] shows a molecular ion peak at 416m/z, The other peaks are shown as follows

- [Cu (L) Cl] $^+$ = 381 m/z
- [Cu (L)] $^+$ = 345 m/z

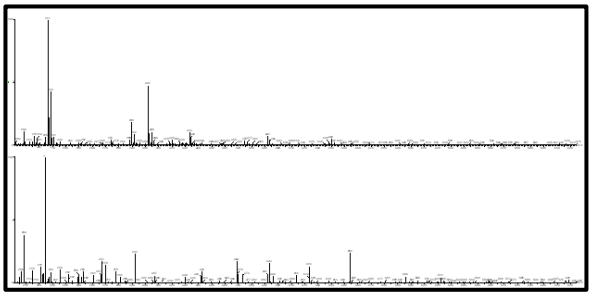

Fig. 9. MS Spectra of the Cu Complex

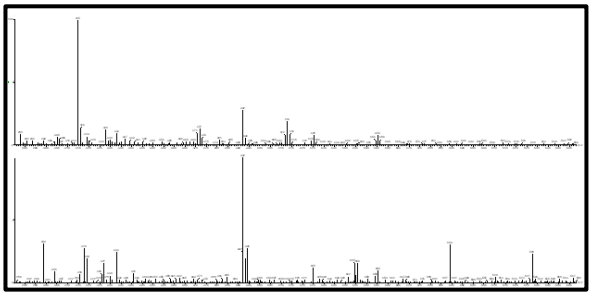

Fig. 10. MS Spectra of the Fe Complex

The complex [Cr $(L)_2$ Cl_2] Cl showed a molecular ion peak at [M0] = 723 m/z which is equivalent to molecular mass of the complex. The other peaks are shown as follows.

- [Cr $(L)_2$ Cl_2] = 687 m/z
- [Cr $(L)_2$ Cl] = 652 m/z
- [Cr $(L)_2$] = 616 m/z
- [Cr (L)] = 334 m/z

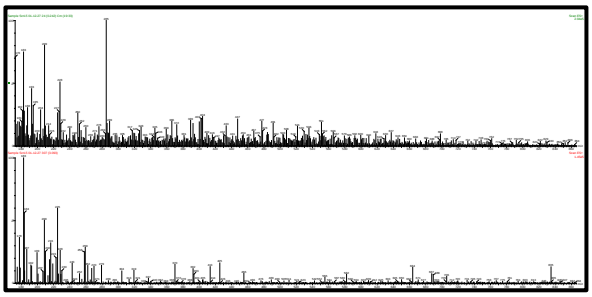

Fig. 11. MS Spectra of the Cr Complex

Antioxidant Activity Study

The scavenging activity results of synthetic compound:

scavenging activity % in (12.5-100) µg/ml is (29%-78 %)

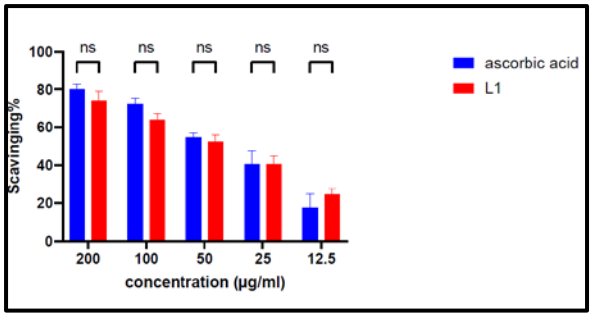

Fig. 12. scavenging activity assay by DPPH of compound L_1

L1: 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2yl)hydrazinylidene]methyl}phenol
The conc. (12.5) µg/ml is the most scavenging activity compared with other concentrations of [2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2yl)hydrazinylidene]methyl}phenol] because it's scavenging activity is more than scavenging activity of ascorbic acid in this concentration [26-28].

Anti-cancer activity
The new ligand [2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2yl)hydrazinylidene]methyl}phenol] has a high anti-cancer activity Breast cancer was analyzed for the prepared sample, where we found the prepared compound has a high effect on cancer cells and a mild effect on normal cells,.

And when testing the ligand on a breast cancer cell line, it was found to have a high effectiveness, as the percentage of inhibition on the MCF-7 cell line ranged between (56.79-4.83) at concentrations (12.5-400). While it did not show high cytotoxicity when tested on normal cells, as the percentage of inhibition ranged between (28.05 - 4.06) at concentrations (400 - 12.5). as shown in the following (Table 3).

Also, the results showed significant differences, $P \leq 0.0001$, when calculating the (IC50) when treating the fourth ligand for MCF-7 cancer cells (110.6 µg/ml) and for normal cells WRL68 (236.0 µg/ml), as shown in the following (Table 3)(Figure 12, 13).

Tab 3. The cytotoxic effect of CuL1 on WRL68 and MCF-7 cell line.

Concentration µg mL^{-1}	**Mean viability (%) ± SD**	
	WRL68	**MCF-7**
400	71.95±0.81	43.21±2.43
200	84.79±1.20	49.57±4.99
100	93.59±2.10	73.03±4.64
50	95.33±1.18	90.70±3.18
25	95.21±0.82	95.71±0.8
12.5	95.949±1.02	95.17±1.2

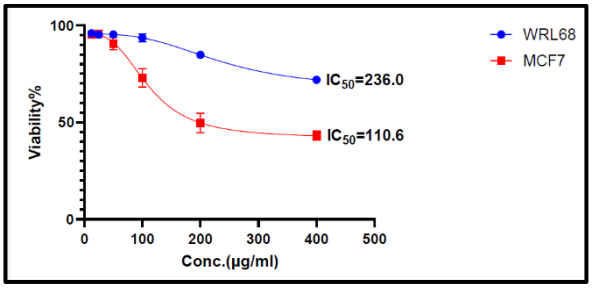

Fig 13. The cytotoxic effect of CuL1 on WRL68 and MCF-7 cell line.

CONCLUSION

The new ligand 2-methoxy-4-{(E)-[2-(5-sulfanyl-1,3,4-thiadiazol-2yl)hydrazinylidene]methyl}phenol the spectroscopic data display the involvement of CH=N groups in coordination to the central transition metal ion. According to hyper chem characterization of transition metal complexes shows that octahedral geometry for Fe (III), Cr (III), tetrahedral geometry for Cu (II), and square planar geometry was suggested for Ni (II). (L) was successfully synthesized. It acts like a bidentate ligand and Tri dentate ligand. The ligand gave high efficacy when compared with antioxidant compounds such as vitamin C as well as anti-cancer compounds

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