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Microwave Assisted Synthesis, Characterization and Biochemical Study of New Chalcones.

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Abstract

A number of new chalcones were prepared from 1,2-bis(2-methoxy-4-vinylphenoxy) ethane by Claisen–Schmidt condensation using a microwave as a heat source, which has beneficial effect on many important aspects such as reducing reaction time, lowering the solvent volume, increasing yield and purity improvement. The synthesized chalcones were characterized on the basis of their chemical properties and spectroscopic data (FTIR, ^{1}H NMR, ^{13}C MNR and CHNO-S). Their biological activities were also investigated and found that some are good anticancer and antioxidants when they subjected to *in vitro* cytotoxicity test.

Keywords: Chalcones, Vanillin, Microwave, Hepatoxicity, Anticancer.

1. Introduction

Chalcones recently have received considerable attention in medicinal chemistry [1]. The natural or synthetic chalcones are important compounds not only because of their biological properties but also because they serve as important intermediates for the synthesis of a large number of heterocyclic systems [2-4]. Moreover, some of these derivatives have been found to inhibit several important body enzymes in cellular systems including, aldose reductase, epoxide hydrolase [5] and xanthine oxidase [6].

Moreover, the Microwave assisted in chemistry synthesizes have significant advantages comparted with conventional methods, such as reducing the reaction time, solvents and that be useful from the commercial side beside the products be more purity. [7,8]

chalcones have been extensively studied for their biological activities, including bacteriostatic [9], antitumor [10], fungistatic [11], antiparasitic [12], cardiovascular [13], anticancer [14], anti-inflammatory [15], antitubercular [16], and antifung [17] activities. In fact, the pharmacological properties of Chalcones are belong to the presence of both α,β-unsaturation and an aromatic ring. [18-21]

2. Materials

Vanillin, dibromoalkane, 4-chloro acetophenone, 4-bromo acetophenone, 4-methoxy acetophenone, 4-nitro acetophenone, 4-amino acetophenone and potassium carbonate were supplied by Sigma-Aldrich. Acetophenones and hydrochloric acid were supplied by Fluke. 4-methyl acetophenone obtained from B.D.H. While, DMF and ethanol were supplied by Merck and S.C.H, respectively. All materials used in current study without purification.

3. Experimental

3.1 Synthesis of compounds

3.1.1 Synthesis of 1,2-bis(2-methoxy-4-vinylphenoxy) ethane

Vanillin (10.0 mmol, 1.50 g), dibromoalkane (4.0 mmol), potassium carbonate (3.0 g, 25.0 mmol), PEG-400 (0.5 g), and DMF (3.0 mL) were charged into 50 mL flat bottom flask. Then, the mixture was subjected to microwave irradiation for 2–3 min (390 W). After that, the mixture was cooled to room temperature and the product was precipitated by adding 50 mL of water, filtered, washed three times and recrystallized

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from ethanol. White solid powder was finally obtained with yield of 87% [22], as shown in Fig. 1.

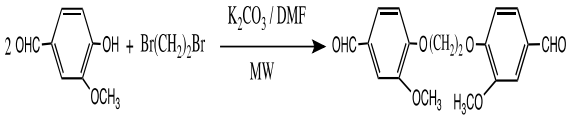
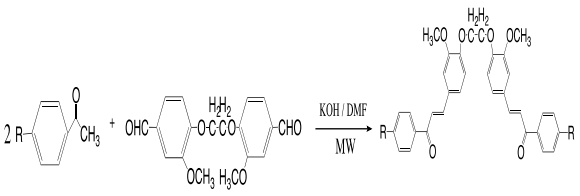


Fig. 1. Synthesis 1,2-bis(2-methoxy-4-vinylphenoxy) ethane from Vanillin compound

3.1.2 Synthesis of Chalcones

A mixture of (0.01 mole) of 1,2-bis(2-methoxy-4-vinylphenoxy) ethane and (0.02 mole) substituted Acetophenones were dissolved in 3 ml of DMF and 1 ml of (40%) KOH and then, irradiated by microwave 90 W for 2-7 min, with continuous reaction monitoring by TLC. Next, it was poured into crushed ice and acidified with (1 N) HCl. The solid was separated, filtered and recrystallized from absolute ethanol. The products were obtained in 70-88% yield. Physical properties of chalcones compounds are illustrated in Table (1) and chemical structures was also shown in Fig. 2. [22,23], and there was significant different in reaction time, solvent volume and production purity comparted with conventional method [24].



R = H, CH_3, OCH_3, Br, Cl, NH_2, NO_2

Fig. 2. Synthesis of Chalcones from 1,2-bis(2-methoxy-4-vinylphenoxy) ethane.

3.2 Experimental enamels

Eighteen male rats, with weigh of (300 – 330) gm and age of (10- 14) weeks, were randomly divided into three groups (6 rats in each group) as the following;

Group I (control group): Received normal saline (2 ml/Kg) orally.

Group II: Injected intra peritoneal (I.P) with CCl_4 (2 ml/Kg) at every 72 h for 10 days.

Group III: Received compound (50 mg/Kg) orally for 10 days and simultaneously administered CCl_4 (2 ml/Kg, I.P) at every 72 h [25].

Blood was collected from the heart by 10 ml disposable syringe. One ml of blood was transferred into EDTA tube for haematological measurements, the remaining volume of blood transferred into a plain tube and centrifuged at 3000 rpm for 15 minutes to obtain the serum which then transferred into Eppendorf tubes and stored at – 20℃ till used for measurement liver enzymes.

Blood parameters were measured by using haematology analyzer count 60, which included a number of red blood cells (RBC), haemoglobin concentration (Hb) and number of white blood cells (WBC). While, the lever enzymes measured according to literature [26].

3.3 Maintenance of cell cultures

CAL51 cancer cell line was obtained from the IRAQ Biotech Cell Bank Unit in Basrah and maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 unit/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 50% confluence twice a week and incubated at 37 °C and 5% CO_2 [27].

3.4 Combination Cytotoxicity Assays

To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates. Cell line CAL51 were seeded at 1×10^4 cells/well. When confluent monolayer was achieved, typically after 24 h, cells were treated with the tested compound with final concentration 1000 µg/ml. Cell viability was measured after 72 h of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT and incubating the cells for 2 h at 37 °C. After removing the MTT solution, the remaining crystals in the wells were solubilized by the addition of 100 µL of DMSO (dimethyl sulphoxide), followed by 37 °C incubation for 15 min with shaking [28]. The absorbency was determined on a microplate reader at 620 nm (test wavelength); the assay was performed in triplicate. The inhibition rate (IR) of cell growth (the percentage of cytotoxicity) was calculated as the following equation [31].

$$\textit{Proliferation rate } \boldsymbol{(PR)= B/A*100} \quad (1)$$

$$\boldsymbol{IR= 100- PR} \quad (2)$$

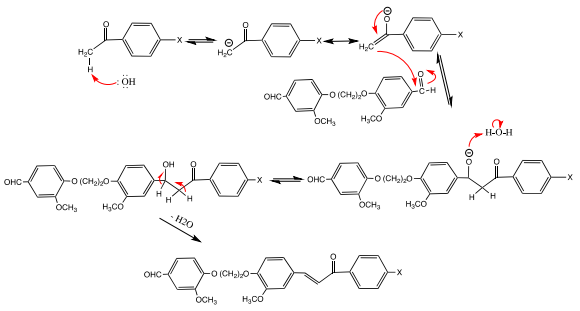
Where A is the mean optical density of untreated wells and B is the optical density of treated wells.

Table 1. Some Physical Data of the synthesized chalcones derivative from 1,2-bis(2-methoxy-4-vinylphenoxy) ethane

Symbol Chalcones	Name of Chalcones	Color	Melting Point (^{0}C)	Yield (%)
X1 (4-Br)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-bromophenyl)prop-2-en-1-one)	Yellow	155-157	88
X2 (4-CH3)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-methylphenyl)prop-2-en-1-one)	Yellow	110-112	81
X3 (4-Cl)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-chlorophenyl)prop-2-en-1-one)	Yellow	160-162	88
X4 (H)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-phenylprop-2-en-1-one)	Yellow	125-127	83
X5 4-NH2	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-aminophenyl)prop-2-en-1-one)	Pale yellow	217-220	86
X6 (4-NO2)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-aminophenyl)prop-2-en-1-one)	Dark yellow	193-195	90
X7 (4-OCH3)	3,3'-((ethane-1,2-diylbis(oxy))bis(3-methoxy-4,1-phenylene))bis(1-(4-methoxophenyl)prop-2-en-1-one)	Yellow	113-115	83

4. Results and Discussion

Microwave irradiation was implemented to react 1,2-bis(2-methoxy-4-vinylphenoxy) ethane and substituted acetophenones. The mechanism of reaction was illustrated in Fig. 3. It showed that the nucleophilic attack of enolate anion at carbon atom of carbonyl related to 1,2-bis(2-methoxy-4-vinylphenoxy) ethane with elimination of water at the end of the reaction.



X = H, CH_3, OCH_3, Br, Cl, NH_2, NO_2

Fig. 3. Mechanism Formation Chalcone Compounds

4.1 *Characterization of compounds*

The synthesized chalcones and heterocyclic compounds were characterized by elemental analysis (CHNO-S), and their spectra were recorded by Eager 300 for EA1112 Analyzer. It was found that the calculated values of carbon, hydrogen and nitrogen elements are compatible with observed value which confirmed the validity of the suggested structure of the synthesized compounds. See Table 2.

Table 2. Elemental Analysis of Chalcone Compounds

Sym.	Molecular formula	Calculated			Observed		
		%C	% H	% N	%C	% H	% N
X1 (4-Br)	C34H28Br2O6 691.604	59.04	4.08	-	58.84	4.15	-
X2 (4-CH3)	C36H34O6 562.66	76.84	6.09	-	76.77	6.11	-
X3 (4-Cl)	C34H28Cl2O6 603.49	67.67	4.67	-	67.59	4.61	-
X4 (H)	C34H30O6 534.60	76.39	5.65	-	76.42	5.59	-
X5 4-NH2	C34H32N2O6 564.63	72.32	5.71	4.96	72.43	5.66	4.83
X6 (4-NO2)	C34H28N2O10 624.59	65.38	4.52	4.48	65.33	4.59	4.44
X7 (4-OCH3)	C36H34O8 594.65	72.71	5.76	-	72.67	5.86	-

FTIR Spectra of chalcone compounds were recorded by SHIMADZU FTIR-8400 S instrument. All spectra indicate appearance of absorption band ranged from 1665 cm^{-1} to1710 cm^{-1} related to the α,β unsaturated (C=O) stretching. The IR spectra of X5 showed a strong band between of 3300 cm^{-1} and 3500 cm^{-1} assigned to N-H stretching. Also, strong bands appeared between 1527 cm^{-1} and 1600 cm^{-1}, which were characteristic of all chalcone compounds, related to the (C=C) stretching of aromatic ring. Other medium and week absorption bands at ranged from 3000 cm^{-1} to 3100 cm^{-1} and 2916 cm^{-1} to 2978 cm^{-1} are corresponding to aromatic and aliphatic (C-H) stretching, respectively. Moreover, two absorption bands, at 3310 cm^{-1} and 3500 cm^{-1}, assigned to stretching of (NH_2) group. X1 and X3 contain (C-X) bond which appears as a strong absorption band between 698 cm^{-1} and 825 cm^{-1}. Table 3 also shows detailed information of absorption bands of compounds (X1-X7). These results are in conformity with the literature information [30-34].

Table 3. Data of the FT-IR Spectra of chalcone Compounds

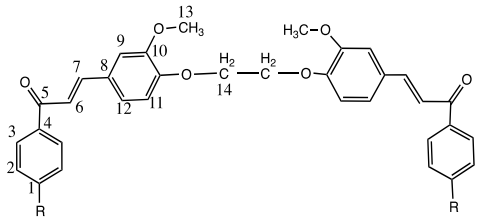
Sym.	υNH Str. cm-1	υC=O Str. cm-1	υC=C Ar. Str. cm-1	υC=C Al. Str. cm-1	υCH Ar. Str. cm-1	υCH, CH3 Al. Str. cm-1	υC-H al cm-1
X1	-	1675s	1509s	1588s	3053m	2934m	823s
X2	-	1681s	1597	1651	3061m	2881m	-
X3	-	1681s	1512s	1589s	3070m	2935m	813s
X4	-	1673s	1509s	1590s	3060m	2936m	-
X5	3450m 3356	1634s	1510m	1594s	3067m	2927m	
X6	-	1685s	1512s	1587	3080m	2855m	
X7	-	1682s	1513s	1595s	3051m	2949m	-

Further characterization of the chalcone compounds was made by NMR analysis using AC-400MHz SHIMAZU spectrometer and deuterated solvent of DMSO-d_6. The frequency was adopted to 400 MHz for ^{1}H NMR and to 100 MHz for ^{13}C NMR. The ^{1}H NMR spectra of the chalcone compounds were indicated presence four types of protons; aromatic ring protons which showed signals between 6.42 ppm and 8.53 ppm, (C=CH) protons that neighbor the protons of aromatic ring appear signal between 7.45 ppm and 8.5 ppm, protons of methoxy groups which showed singlet signal at the chemical shift between 3.5 ppm and 3.89 ppm, and protons of methylene group (-CH2-) exhibited triplet signal ranged from 4.10 ppm to 4.48 ppm [37-39]. However, additional signals were found in X2 and X5, (6.00-6.11 ppm) and 2.5 ppm, belonged to protons of methylene group and two-protons of primary amino group, respectively [38-41]. The chemical shifts of these compounds are also summarized in Table 4.

Table 4. ^{1}H NMR Chemical shifts (ppm) of chalcone compounds.

Sym.	Ar. =CH	NH_2	CH_2	OCH_3	CH_3
X1	6.50-8.10	-	4.15	3.82	-
X2	7.00-8.05	-	4.48	3.87	2.5
X3	6.76-8.17	-	4.24	3.80	-
X4	6.75-8.15	-	4.48	3.76	-
X5	6.42-7.78	6.11	4.48	3.88	-
X6	7.26-8.36	-	4.48	3.87	-
X7	6.92-8.53	-	4.48	3.84	-

^{13}C NMR spectrum of the all compounds showed signals at the chemical shift between 50 ppm and 70 ppm ascribed to the carbons of OCH_3 and OCH_2. More, signal between 185 ppm and 195 ppm belonged to carbon of (C=O) group. The aromatic ring carbons were indicated by appearance of signal within the range of 129 ppm to 146 ppm. The multi-peaks between 43 ppm and 45 ppm related to carbon of DMSO solvent [42-47]. Other signal was found in X2 at chemical shift of 21 ppm and assigned to carbon of methylene group [48]. ^{13}C NMR chemical shifts were also listed in Table 5 and number of carbon atom of chalcone was illustrated in Fig. 4.



R = H, CH3, NH2, NO2, Br, Cl, OCH3

Fig. 4. Numbering carbon atoms of Vanillin chalcone

Table 5. Chemical Shift (ppm) of Vanillin chalcone compounds

C Atom	X1	X2	X3	X4	X5	X6	X7
C1	127.93	143.78	138.54	133.53	153.98	153.57	158.40
C2	132.18	129.06	129.16	128.40	113.15	126.37	112.99
C3	130.43	129.36	130.20	129.09	131.48	130.41	130.41
C4	136.20	135.76	135.83	137.28	126.01	137.40	130.16
C5	191.80	191.86	191.74	191.77	196.32	191.86	191.89
C6	120.28	124.21	126.32	124.31	123.63	124.26	123.97
C7	146.38	143.78	145.39	144.94	143.73	149.63	144.87
C8	126.38	130.41	130.26	126.36	126.01	126.37	130.16
C9	110.22	110.26	110.18	111.54	111.29	110.26	110.02
C10	149.07	153.57	149.13	149.57	150.00	153.57	149.50
C11	112.74	112.87	119.94	113.19	118.83	112.88	114.84
C12	120.28	120.30	124.44	120.26	123.63	124.26	122.67
C13	55.89	55.97	55.88	55.91	56.19	55.97	55.74
C14	67.78	67.65	67.62	67.77	67.80	67.65	67.56
C15	-	21.64	-	-	-	-	-

From the mass spectra found that the peaks at (m/z = 562, 603, 534, 564, 624 and 594) represented the molecular ion [M+] for (X2-X7) compounds, respectively. These peaks support our study while the

structures of the chalcone compounds that were synthesis is correct, as show in Fig. 1S – Fig. 6S.

4.2 Biochemical sudy

4.2.1 haematological study of compound (X5)

The effect of chalcone compound (X5) on RBCs counts, haemoglobin and WBCs counts in hepatotoxicity male rats was induced by CCl_4. The results showed a high decrease ($p < 0.001$) in RBCs counts and haemoglobin concentration and a high increase ($p < 0.001$) in WBCs counts after treatment with CCl_4 as compared with control group. Comparable results were obtained after treatment with chalcone compound, which exhibited a significant increase ($p < 0.001$) in RBC counts and haemoglobin concentration as well as significant decrease ($p < 0.001$) in WBC counts, as shown in Table 6 and Fig. 5.

Table 6. Effect of Chalcone compound (X5) on RBCs counts, Haemoglobin concentration and WBCs counts in hepatotoxicity male rats induced by CCl_4

Parameters / Treatments	N	RBC×10^6 / µL	Hb g/dL	WBC ×10^3/µL
Control (Normal Saline)	6	8.30±0.39	14.00±0.37	7.37±0.40
CCl_4	6	5.24±0.42 ***	8.46±0.43 ***	10.78±0.56 ***
CCl_4+Chalcon compound (50 mg / Kg)	6	7.71±0.28 ***	12.47±0.48** **	7.18±0.62 ***

N= number of animals, Mean ±SD, * p≤ 0.05, **p ≤ 0.01, ***p≤ 0.001

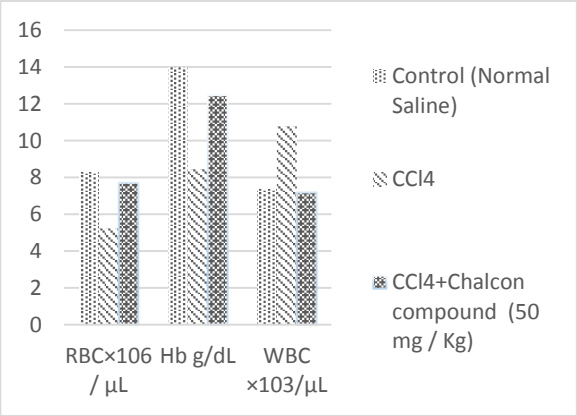


Fig. 5. Effect of Chalcone compound (X5) on RBCs counts, Haemoglobin concentration and WBCs counts in hepatotoxicity male rats induced by CCl_4.

This results come with agreement of literature [49]. The decreasing in RBCs count and haemoglobin concentration may be attributed to the oxidative stress produced by CCl_4 [50] and hemolytic anemia when sulfhydryl groups of the erythrocyte membrane are oxidized, which inflicts injury to the erythrocytes membrane and may be due to destruction of hematopoiesis and reduction in the rate of formation of RBCs. Other observation involves an increasing in WBCs count and that may attribute to the defensive mechanism of the immune system [51].

The amelioration of toxic effect of CCl_4 after treatment with chalcon compound may attributed to unsaturated double bounds which gave it antioxidant activity by inhibition of lipid peroxidation in the erythrocytes membranes and resistance to hemolysis [52]. The decrease in WBCs after treatment with chalcon compound may be relating to the anti-inflammatory activity by presence methoxy groups [53, 54].

4.2.2 hepatotoxicity study

The effect of chalcone compound X5 on liver enzymes in hepatotoxicity male rats was also induced by CCl_4 and the obtained results were listed in Table 6. By comparing with control group, it revealed a high increase ($p < 0.001$) in serum level of ALT, AST and ALP after treatment with CCl_4. While, lower serum level ($p < 0.001$) was observed after treatment with chalcone compound X5 as compared with CCl_4 treatment group, as shown in, Table 7 and Fig. 6.

Table (7) Effect of chalcone compound (X5) on Liver enzymes in hepatotoxicity male rats induced by CCl_4.

Parameters / Treatments	N	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control (Normal Saline)	6	48.07±0.83	45.40±0.47	40.36±0.68
CCl_4	6	78.33±0.49	70.96±0.75	74.66±0.64
CCl_4+Chalcon compound (50 mg / Kg)	6	49.06±0.81	52.65±1.36	52.53±0.99

N= number of animals, Mean ±SD, * p≤ 0.05, **p ≤ 0.01, ***p≤ 0.001

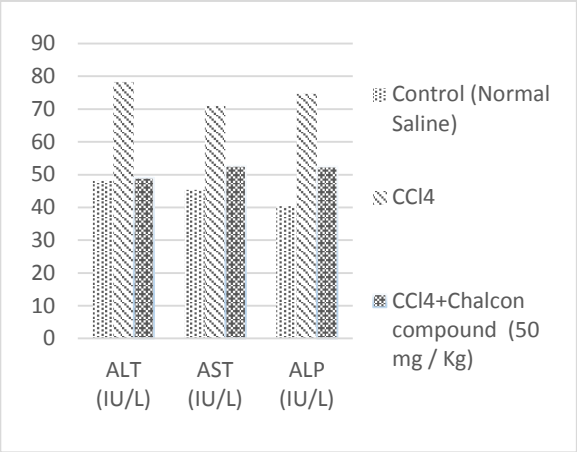


Fig. 6. Effect of chalcone compound (X5) on Liver enzymes in hepatotoxicity male rats induced by CCl_4.

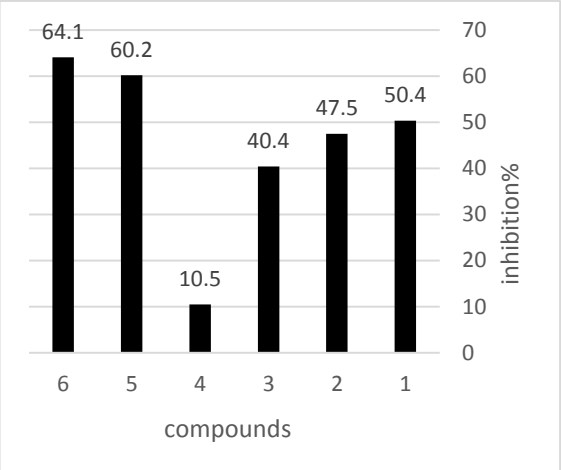


Fig. 7. Rate of inhibition for chalcones compounds on CAL51 cell line.

Over production of reactive oxygen species induces oxidative stress and that result in cellular membrane damage with a consequent alteration in metabolic processes. Reactive oxygen species play a crucial role in the pathogenesis of different human disease such as liver disorders. CCl_4 is a common hepatotoxic used for the induction of liver disorder [49]. This effect is due to the release of free radicals which are composed of trichloromethyl (CCl_3) and peroxy trichloromethyl ($OOCCl_3$) radicals [55]. These free radicals can generate lipid peroxide which may cause cell membrane damage, alteration in enzyme activity and induction of hepatic injury and necrosis [56]. It was observed that elevated levels of liver enzymes due to the altered permeability of the cell membranes, the enzymes leak into the circulatory system and show intense injury to the liver structure. While, it also noticed that a significant decrease in enzymes levels after treatment with chalcone compound X5 [57]. The chalcone contains substituted- methoxy groups and double bond, i.e. X5 compound, significantly enhanced anti-inflammatory and anti-oxidant activity.

4.2.2 cytotoxicity study

The *in vitro* cytotoxicity assay of chalcone compounds indicated that compounds (X2, X4 and X5) have the highest inhibition rate (64.1, 60.2 and 50.4), respectively. While, compounds (X6, X1 and X7) have the lowest inhibition rate (47.5, 40.4 anf10.5), respectively, as shown in Fig. 7.

The activity of chalcone compounds as anticancer might attribute to induction of apoptosis, DNA and mitochondrial damage. Some of clinically useful anticancer drugs have genotoxicity as a result of their interaction with amino groups in nucleic acids-chalcones which, consequently, devoid side effects and improves structural flexibility [58]. The relationship between cytotoxicity with chalcone structure was also studied and found that ring-substituted methyl, hydrogen and amine increases the activity of cytotoxicity and this come with agreement of study which correlated the anticancer activity of amino chalcone with the presence of amino group [59].

5. Conclusion

In this study, a number of new chalcones were synthesized from the various acetophenon substituted-1,2-bis(2-methoxy-4-vinylphenoxy) ethane by Claisen–Schmidt condensation and microwave technology. Possible reaction mechanisms were considered, and chemical structures of the new products were confirmed, based upon compatible elementary and spectroscopic evidence (FTIR, ^{1}H NMR, ^{13}C MNR and CHN analysis). The effect of chalcone compound X5 on RBCs counts, Haemoglobin and WBCs counts in hepatoxicity male rats was induced by CCl_4 and good biological activates were obtained. Additionally, *in vitro* anticancer activities of these compounds were evaluated against

human breast cancer cell line Cal51, and compared with standard anticancer drugs, employing standard MTT assay in which compounds X2, X4 and X5 showed the highest inhibition rate, 64.1, 60.2 and 50.4, respectively. While, compounds X1, X6 and X7 exhibited the lowest inhibition rate, 47.5, 40.4 and 10.5, respectively.

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