Open Access
Baghdad Science Journal **Vol.16 (4) Supplement 2019**

DOI: http://dx.doi.org/10.21123/bsj.2019.16.4(Suppl.). 1003

Synthesis, Cytotoxicity, Xanthine Oxidase Inhibition, Antioxidant of New Pyrazolo{3,4 d}Pyrimidine Derivatives

Sameaa J. Khammas ***Ahlam J. Hamood***

Received 25/3/2018, Accepted 30/5/2019, Published 18/12/2019

Abstract:

Allopurinol derivative were prepared by reacting the (1-chloroacetyl)-2-Hydropyrazolo{3,4-d}pyrimidine-4-one with 5- methoxy- 2-amino benzothiazole under certain conditions to obtain new compound (N- (2-aminoacetyl (5-methoxy) benzothiazole -2yl) (A_4), Reaction of 5-(P-dimethyl amine benzene)-2-amino-1,3,4- oxadiazole in the presence of potassium carbonate anhydrous to yield new compound (N-(2- aminoacetyl-5-(P-dimethyl amine benzene)-1,3,4-oxadiazoles-2-yl)(A_{30}) and Azo compound (N-(5-(Azo-2-hydroxy-5-amino benzene)-1,3-Diazol-2yl)Allopurinol(A_{46}). The structure of prepared compounds were confirmed by (FT-IR) technique and their physical properties. The synthesized compounds were tested for cytotoxicity, Xanthine oxidase inhibition, and antioxidant activity.

Key words: Allopurinol, Antioxidant Activity, Azo compound, Cytotoxicity, Xanthine Oxidase.

Introduction:

Allopurinol is a polar compound with strong intermolecular hydrogen bonding and limited solubility in both polar and non-polar media .It is a very weak acid with a dissociation constant (pka) of 9.4 and is therefore essentially unionized at all physiological PH values.

Allopurinol is an inhibitor of the enzyme commonly known as xanthine oxidase(1). Allopurinol is an analogue of hypoxanthine. It is effective for the treatmen of both primary hyperuricemia of gout and secondary hyperuricemia related to hematological disorders or anti-neoplastic therapy.

Allopurinol an isomer of hypoxanthine and its active metabolite oxipurinol (alloxanthine) act by inhibiting xanthine oxidase, an enzyme which forms uric acid (urate) from xanthine and hypoxanthine(2).

Metabolic activity can be evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. MTT is designed for the quantification of cytotoxic index in cell population using 96 well plate format(3).

The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol -2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water.

Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage cannot transform MTT(4).

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical anion, hydroxyl radical, alkyl peroxyl radical, nitric oxide, and singlet oxygen are often associated with some physio pathological states in human. Oxidative stress caused by an imbalance between antioxidant systems and the production of oxidants including ROS contribute to a wide variety of degenerative processes and diseases such as atherosclerosis, Parkinson's disease, Alzheimer's dementia, and reperfusion injury of brain or heart and also can be associated with the pathogenesis of various conditions such as aging, arthritis, cancer, and inflammation(5).The Aim of Work;search aims at preparing new derivatives of drug (Allopurinol derivatives) and studying the Cytotoxicity, Inhibitors enzyme (Xanthine oxidase) and antioxidant of new derivatives .

Materials and Methods:

Chemicals:

1- All chemicals used were supplied from Merck, GCC companies, BDH, Fluka, Sigma Aldrich.

Department of Chemistry, College of Science for Women, University of Baghdad, Baghdad, Iraq.
*Corresponding author: sjk_1975@yahoo.com

2- Cytotoxicity contained: - (Fetal Bovine Serum Gold made of Austria., Rpmi Medium+ GlutaMax made of ,LSM Lymphocyte made in Austria) .
3- Xanthine oxidase kit (Abonova , Assay kit) made in Taiwan.
4- Total Antioxidant Capacity (Cohesion, Micro plate Assay Kit) made in UK.

Instruments

1- Melting points were determined on Sturat Scientific melting point SMPLU-K and were uncorrected.
2- Infrared spectra (FT-IR) were recorded using KBr disk on shimadzu FT-IR-8400 spectrophotometer in Ibn Sina State Company (ISSC).
3- Concentration of enzymes were measured by Elisa (BECKMAN COULTER) made in U.K.
4- Vorter mixer (model VM 300) made inChina.
5- KAN-SHAKER (TKA 226 100S) made in China.
6- Platelet centirfuge (PC-810) made in Japan.
7- Incubator (gallenkamp, Economy incubator with size 2) made in Germany.
8- High speed centrifuge (eppendrof) made in Germany.
9- Microscope Micropipette (Humapette -8(30-300 µl)) made in France.
10- (MEIJI TECHNO, TC5400) made in Japan.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to affect different factors in study parameters . Least significant difference –LSD test (ANOVA) was used to compare between means in this study .

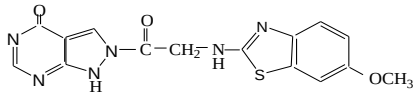
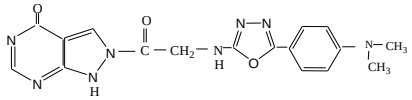
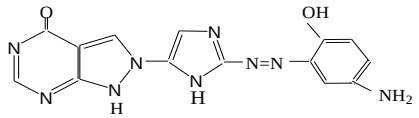
Experimental

Synthesis of 2-[2-aminoacetyl (5- methoxy) benzothiazole] Allopurinol (A_4) 2-Chloroacetyl Allopurinol(A) (0.008 mol) in absolute ethanol (25 mL) and potassium carbonate anhydrous (0.008 mol) was refluxed (8 hours) and added dropwise to a solution of (0.008 mol) of 2-amino(5- methoxy) benzothiazole dissolved in (30 mL) of absolute ethanol. After cooling, the separated precipitate was filtered and recrystallized from a suitable solvent (absolute ethanol). Physical properties are listed in Table (1)(6).

Synthesis of 2-[2-amino acetyl-5-(P- N,N-dimethyl amino benzene)-1,3,4-oxadiazoles] Allopurinol (A_{30}) (6) 2-Chloroacetyl Allopurinol (1 gm , 0.004 mol) in absolute ethanol (10 mL) and potassium carbonate anhydrous (0.56 gm , 0.004 mol) was refluxed (6 hours) and added dropwise to a solution of (0.004 mol) of 2-amino-5-(P- dimethyl amine benzene) -1,3,4-oxadiazole dissolved in (10 mL) abs. ethanol.The resulted mixture was cooled to room temperature before pouring into crushed ice. The obtained precipitate was filtered, washed thoroughly with water and dried then was purified by recrystallization from a suitable solvent. Physical properties are listed in Table(1).

Preparation of Azo compound 2-[2- (Azo-2-hydroxy-5-amino benzene) imidazole -5-yl] Allopurinol (A_{46}) (7,8,9) (2-amino-2-Chloroacetyl Allopurinol)-1,3- Diazole (0.02 mol) the mixture of concentrated hydrochloric acid and water were dissolved (the first solution). The solution was cooled until the temperature of the solution falls below 5℃. Dissolve Sodium nitrite (1.378 gm) in (13.8 mL distilled water) (the second solution). It was cooled to 0℃. Then the sconed solution (2-3 mL at a time) added gradually in to the cold first solution with stringing. The temperature was kept between 0-5℃. The solution of sodium hydroxide (1M) and aldehyde (0.02 mol) was added to the mixture the precipitation was observed and filtered, washed several times to (1:1) ethanol:water then let to dry in adesicater. Physical properties are listed in Table 1.

Table 1. Physical properties for compounds

Compd. No	Structure product	Yield %	Color	M.P.℃
A_4		90	Brown	300-302
A_{30}		90	Brown	318-320
A_{46}		85	Dark brown	88-90

Determination of cell cytotoxicity by MTT assay(10)**:** Before Lymphocyte cells were plated in a 96-well-plate with (10^4 – 10^6 cells) of concentration, the cells administered in media containing [RPMI media+10% FBS(Fetal Bovine Serum)]. Then cells were left to adhere for 24

hours, after that exposed to the compounds [Allopurinol(0.46 µg/mL), A_4(0.84 µg/mL), A_{30}(0.63 µg/mL), A_{46}(0.6 µg/mL)] and returned to the incubator for 24 hours. Subsequently, MTT reagent (10 µL) was added directly to the wells. Cells were returned to the incubator for 4 hours. The formation of insoluble purple formazan from yellowish MTT by enzymatic reduction was dissolved in DMSO after the removal of supernatant. The optical density of solution was measured at 620 nm using a microplate reader

$$\text{growth inhibition} = \frac{control-sample}{control}\ X\ 100$$

IC_{50} : is the concentration of drug at which 50% of your target is inhibited .

Detrmination of Xanthine oxidase(4) The Lymphocyte cells administered in media containing [RPMI media+10% FBS(Fetal Bovine Serum)]. Then cells were left to the incubator for 24 hours. After that exposed to the compounds [Allopurinol (0.46 µg/mL) , A_4(0.84 µg/mL), A_{30}(0.63 µg/mL), A_{46}(0.6 µg/mL)] and returned to the incubator for 24 hours, centirfuged plate, supernatant withdrawal and plated 50 µL to two wells in a 96-well-plate . Add 50 µL of Xanthine Oxidase Standard per well in the designated wells on the plate. Then 50 µL were added of freshly mixing (buffer (4.9 mL), Detector (10-acetyl-3,7-dihydroxyphen-oxazine) (50 µL), and HRP (Horseradish peroxidase) (50 µL))], Incubated for 45 minutes at 37°C and readed at 530 nm.

Xanthine oxidase

$$= [\frac{(Adjusted\ sample\ fluorescence)-(y-intercept)}{Slope}] = X$$

Sample dilution

Determination of Total Antioxidant capacity: The Lymphocyte cells administered in media containing [RPMI media+10% FBS(Fetal Bovine Serum)], then cells were left to the incubator for 24 hours. After that exposed to the compounds [A(0.46 µg/mL), A_4(0.84 µg/mL), A_{30}(0.63 µg/mL), A_{46}(0.6 µg/mL)] and returned to the incubator for 24 hours., Centrifuged plate, supernatant withdrawal and plated 10 µL to two wells in a 96-well-plate.Then added 15µL Dye reagent, Mixed for 5 min, measured at 593 nm and recorded the absorbance (OD).

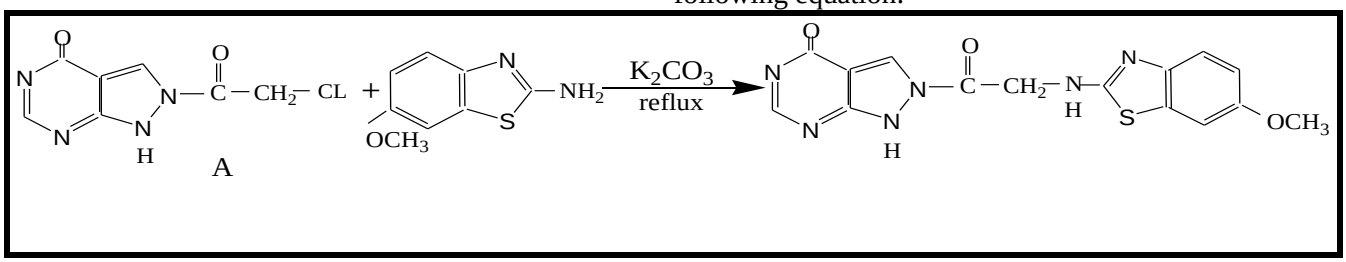
$$\text{TAC } (U/10^4) = \frac{20\ X\ (OD\ sample-OD\ blank) X\ N}{(OD\ standard-OD\ blank)}$$

N: the quantity of cell .

Result and Discussion:

Synthesis of 2-[2-aminoacetyl (5- methoxy) benzothiazol]Allopurinol (A_4)

The reaction of (2-Chloroacetyl Allopurinol)(A) with 2-amino(5- methoxy) benzothiazole in the presence of potassium carbonate anhydrous and absolute ethanol as solvent, as shown in the following equation:



The FTIR of Compound (A_4) (Fig.1) have absorption bands at (3153) cm^{-1} for (N-H), (3082) cm^{-1} (C-H) aromatic, (1583) cm^{-1} (C=O), (1701) cm^{-1} (C=N) ,(1385) cm^{-1} (C-N), (1477) cm^{-1} (C=C) and (C-O) 1230 cm^{-1} .

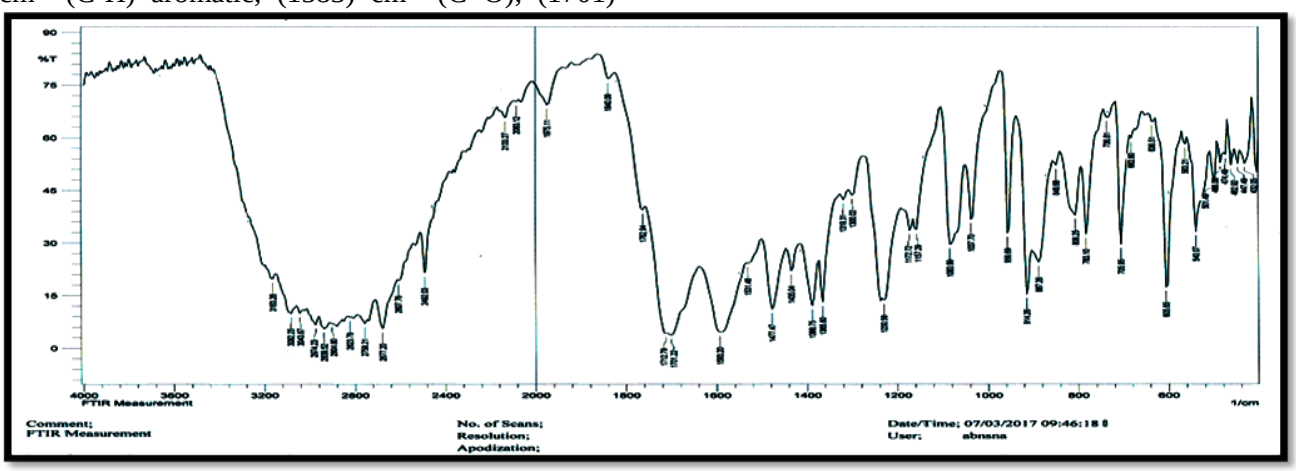
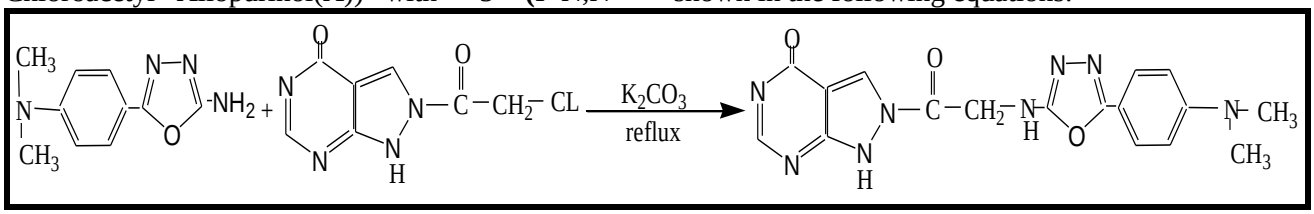


Figure 1. FT-IR Spectral of compound (A_4)

Synthesis of 2-[2-amino acetyl-5-(P- N,N-dimethyl amino benzene) -1,3,4-oxadiazoles) Allopurinol (A_{30}): The reaction compounds (2-Chloroacetyl Allopurinol(A)) with 5- **(**P-N,N-dimethyl amino benzene) -2-amino-1,3,4-oxadiazole in the presence of potassium carbonate anhydrous and ethanol absolute as solvent, as shown in the following equations:



The FTIR of compound (A_{30}) (Fig.2) have absorption bands at (3112) cm^{-1} for (N-H), (1608) cm^{-1} (C=O), (1678) cm^{-1} (C=N) , (1523) cm^{-1} (C=C) aromatic, (1365) cm^{-1} (C-N), (1473) cm^{-1} (N-N) and (1168) cm^{-1} (C-O-C) .

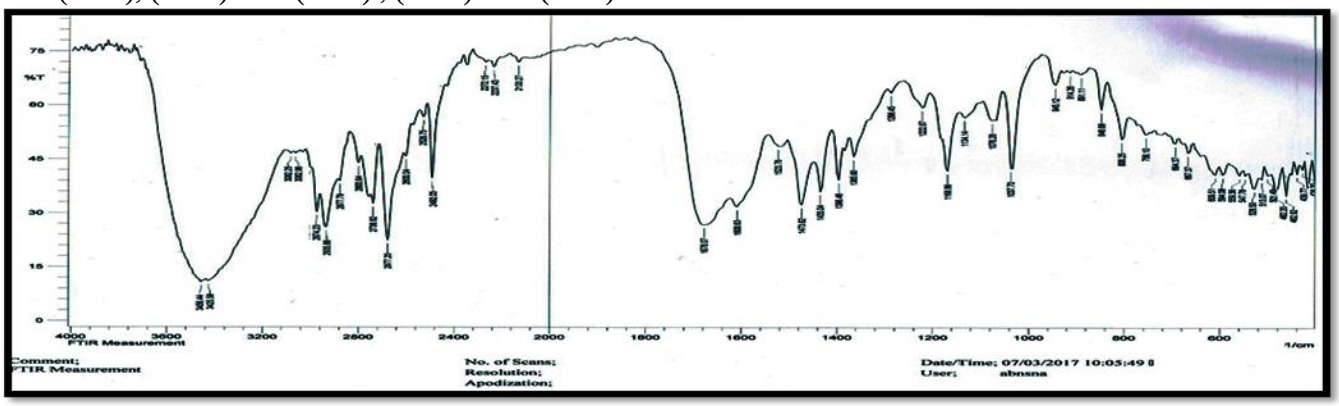
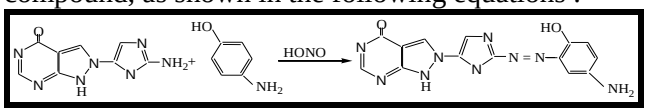


Figure 2. FT-IR Spectral of compound (A_{30})

Preparation of Azo compound (2-[2- (Azo-2-hydroxy-5-amino benzene) imidazole – 5- yl] Allopurinol (A_{46}) :(2-amino-2-Chloroacetyl Allopurinol)-1,3- Diazole was reacted to give Azo compound, as shown in the following equations :



The FT-IR spectra (Fig.3) for compound (A_{46}) shows (N=N) band at (1570) cm^{-1},(C-N) (1315) cm^{-1}, (C=N) (1662) cm^{-1},(NH_2)(3394,3371) cm^{-1}, (C=C) (1570) cm^{-1}, (N-N) (1473) cm^{-1}, (N-H) (3170) cm^{-1}, and (C-H) (3082) cm^{-1}.

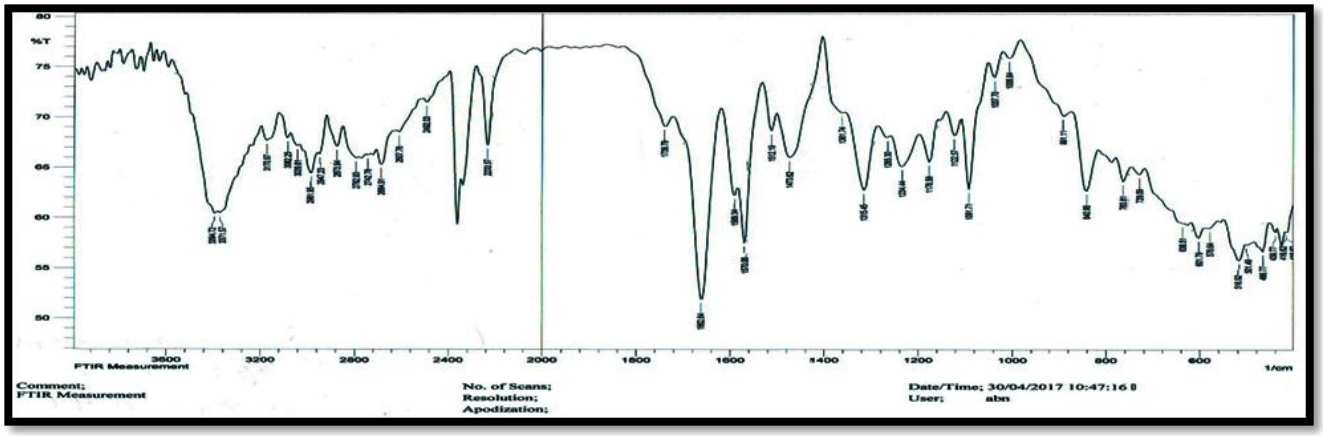


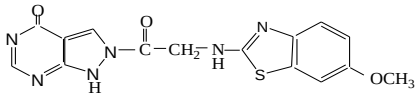
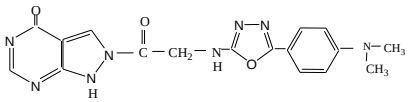
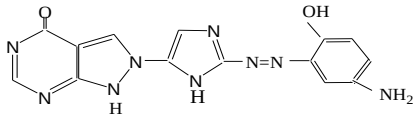
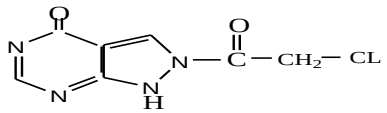
Figure 3. FT-IR Spectral of compound (A_{46})

MTT Assay of Allopurinol Derivatives: The effect of Allopurinol derivatives on lymphocyte cells was estimated through cell toxicity using the MTT assay, analyzing and graphing of the data were carried out by Microsoft Excel 2010 software.

Allopurinol was used as a standard toxicity in assay having IC_{50} (0.46 ± 0.01). The results showed a significant difference ($p<0.05$) in IC_{50} between Allopurinol and its derivatives. There are significant difference ($p<0.05$) between A_{30} (0.63 ±

0.04), A_4 (0.84 ± 0.04) and between A_4 (0.84 ± 0.04), A_{46} (0.60 ± 0.03). There are no significant difference between A_{30} (0.63 ± 0.04) and A_{46} (0.60 ± 0.03). Table 2 show that Allopurinol derivatives have IC_{50} higher than the drug Allopurinol. Those indicated that the derivativs had lower Cytotoxicity against Lymphocyte cells than the drug Allopurinol. The result showed that the substituted Aminobenzothiazol inhibited the viability of Lymphocyte cells lower than substituted oxadiazol and Azo compounds. Electron withdrawing atom substitution such as CL,N in the compounds structure increase the lipopholicity of molecules and is responsible for enhanced Cytotoxcity in MTT assay(4)as shown in Fig. 4 .

Table 2. Cytotoxic effect of Allopurinol derivatives on Lymphocyte cells.

Allop. derivatives	Structure	IC_{50} ± SE(µg/ml)
A_4		*0.84 ± 0.04*
A_{30}		*0.63 ± 0.04*
A_{46}		*0.60 ± 0.03*
Allop.		*0.46 ± 0.01*
LSD value	*0.109*	
	(P<0.05)	

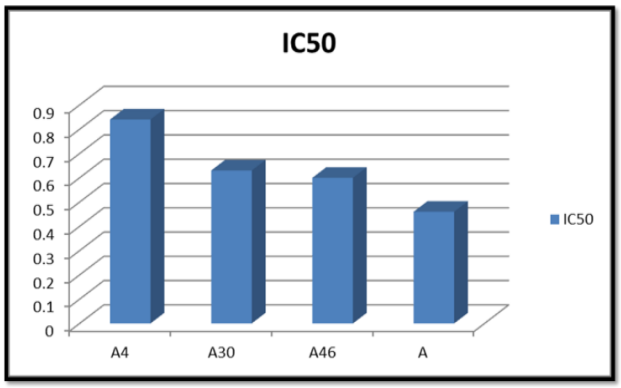
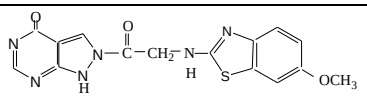
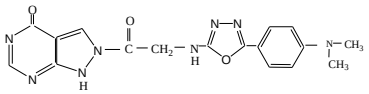
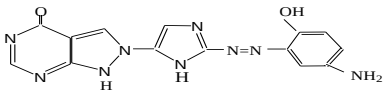
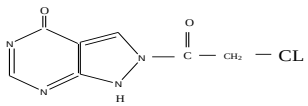


Figure 4. Compare IC_{50} between Allopurinol and Allopurinol derivatives.

Inhibition of Xanthine oxidase activity: The synthesized compounds were tested for their in vitro Xanthine oxidase inhibition. Allopurinol was used as a standard inhibitor in assay having value of (2.61± 0.07) µM. There are significant difference (P<0.05) between A (2.61 ± 0.07) and A_{30} (1.32 ± 0.05), between A_4(2.02 ± 0.05) and A_{30}(1.32 ± 0.05) [104]. There are no significant difference between A (2.61 ± 0.07) and A_4 (2.02 ± 0.05), A_4(2.02 ± 0.05) and A_{46}(2.02 ± 0.06) as shown in Table (3). The result showed that the Allopurinol derivatives are higher inhibition the drug Allopurinol. Among investigated compounds ,A_{30} bearing substituted 1,3,4-Oxadiazol group was found to be the most active Xanthine oxidase inhibitor .Those compounds A_4,A_{30},A_{46} have electron donating at oxygen atom to inhibition the oxidation of Hypoxanthine and Xanthine and more active than the drug Allopurinol(5)as shown in Fig. 5.

Table 3. Inhibitory activity of Allopurinol derivatives on Xanthine oxidase

Allop. derivatives	Structure	Enzyme activity ± SE
A_4		*2.02 ± 0.05*
A_{30}		*1.32 ± 0.05*
A_{46}		*2.02 ± 0.06*
Allop.		*2.61 ± 0.07*
Cont.	*3.92 ± 0.17*	
LSD value		*0.593*
	(P<0.05)	

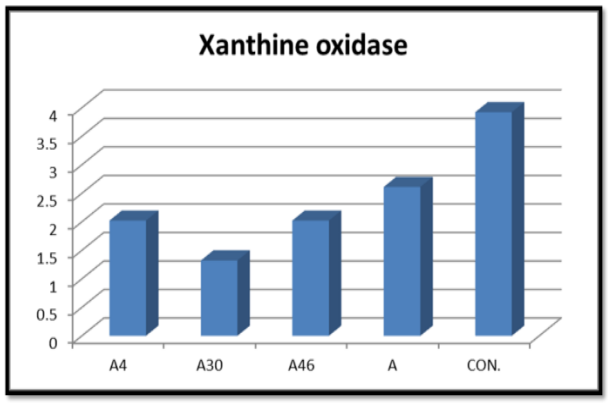
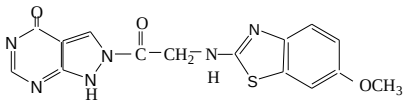
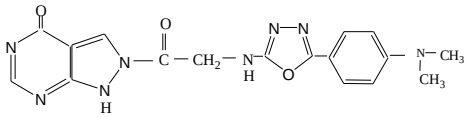
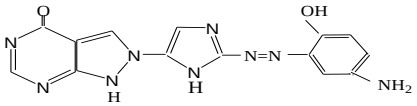
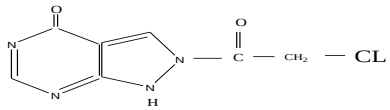


Figure 5. Inhibition of Xanthine oxidase by Allopurinol derivatives.

Effect of Total Antioxidant in the preparation compounds: The synthesized compounds were tested for their in vitro antioxidant as shown in Table (4). There is significant difference ($P<0.05$) between A_4 (521 ± 7.96) and A_{30} (365.00 ± 5.03) , between A_{30}(365.00 ± 0.19) and A_{46}(288.33 ± 3.42). There are no significant difference between A_{30} (365.00 ± 5.03) and control (385 ± 5.71). The most interesting activity was observed in A_4 having substituted aminobenzothiazole group which showed four fold radical scavenging activity as compared to the drug Allopurinol(11)as shown in Fig.6 .

Table 3. Antioxidant activity of Allopurinol derivatives

Allop.derivatives	Structure	Conc.of total antioxidant ±SE
A_4		*521.67 ± 7.94*
A_{30}		*365.00 ± 5.03*
A_{46}		*288.33 ± 3.42*
Allop.		*183.33 ± 4.61*
Control		*385 ± 5.71*
LSD value		*32.803*
	(p<0.05)	

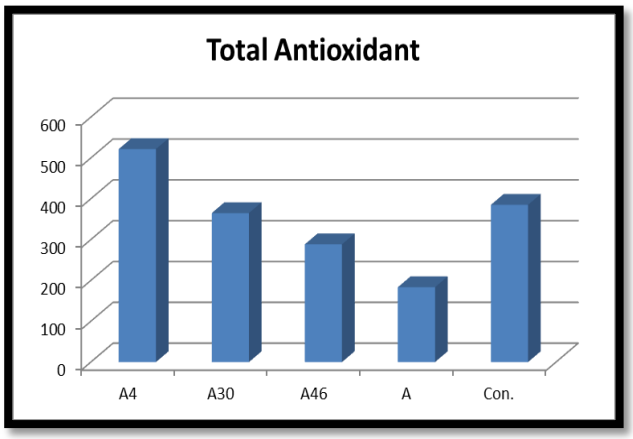


Figure 6. Antioxidant activity of Allopurinol derivatives

Conclusion:

A_4 have low cytotoxicity than Allopurinol, there is no significant difference between A_4 and Allopurinol on inhibition Xanthine oxidase and A_4 have higher antioxidant than Allopurinol .These results indicated that A_4 may be used as drug .

Conflicts of Interest: None.

References:

1. Amal AA, Ahmed MS, Maha AM. Maha KA. Formulation, Characterization and Biopharmaceutical Evaluation of Allopurinol Tablets. I J B. 2011; 2(2) : 63-71.
2. Matteus KR, Lars CN, Eric N VA. simple method for quantification of allopurinol and oxipurinol in human serum by high-performance liquid chromatography with UV-detection. J Ph B A. 2007; 45 (2): 312–317.
3. Leila F, Ronald M, Alexander T. Comparison of Cytotoxic Activity of Anticancer Drugs against Various Human Tumor Cell Lines Using In Vitro Cell-Based Approach. I J B S. 2012; 8 (1): 76-80.
4. Senthilraja P, Kathiresan K. In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF -7 cell lines study of Marine Yeast. J. Applied Pharm. Scie. 2015 ;5(03): 080-084.
5. Danijela AK, Danica SD, Gordana SS, Ivan RP. Xanthine Oxidase: Isolation, Assays of Activity, and Inhibition . Corporation J Ch. 2015; 1-8.

6. Al-Majidi SM. Synthesis, Characterization and Evaluation of Antimicrobial Activity of Several New N-Substituted Carbazole . J Al-Nahrain University. 2013; 16 (4): 67-79.
7. Al-Ajely MS, Al-Ajely HM, Al-Naib AN. Synthesis of Some Substituted 1,2,3-Triazole Derivatives via 1,3-Cycloaddition Reaction of Phenacylazides and Some Substituted Propargyl Compounds . Tikrit J Pure Scie. 2008; 13 (3):1-8.
8. Amer J J, Zaniab S K. Synthesis, spectral and dyeing performance studies of 4-(2-amino-5-nitro-phenylazo)-1,5-dimethyl-2-phenyl-1,2-dihydro-pyrazol-3-one complexes with some metal ions . Baghdad Scie j. 2016; 13(4): 838-845.
9. Issam A M, Asniza M. Synthesis of New Azo Compounds Based on N-(4-Hydroxypheneyl) maleimide and N-(4-Methylpheneyl)maleimide. *Molecule*. 2010; 15(10): 7498-7508.
10. Bushra J M. Msc. Investigate The Different Effect Of Nicotine On H460 and H441 Lung Cells Viability. University of Baghdad; 2017.
11. Muhammed A H, Khurram S, Muhammed S, Nasim H R, Sumera Z, Jamshed I, et al . Synthesis, Urease Inhibition, Antioxidant, Antibacterial, and Molecular Docking Studies of 1,3,4-Oxadiazole Derivatives. I S R N. pharmacology. 2012 Aug; 13: 1- 9 .

تحضير مشتقات جديدة لمركب بايروزولو(d،3,4)بايريمدن وقياس سميتها وقابليتها على تثبيط انزيم الزانثين اوكسيديزوفعاليتها كعوامل مؤكسدة

سميعة جمعة خماس **احلام جميل حمود**

قسم الكيمياء، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق.

الخلاصة:

يتضمن البحث تحضير مشتقات جديدة للالوبيورينول من تفاعل اللوبيورينول مع الكلورواستيل كلورايد بوجود ثلاثي اثيل امين وداي اثيل ايثر كمذيب ليعطي (1- كلورواستيل)-2- هيدروبايرازولو (3,4،د) بايريمدن -4- اون) والذي بدوره يتفاعل مع(5- ميثوكسي) 2- امينو بنزوثايازول تحت ظروف معينة ليعطي المركب (A_4) وكذلك يتفاعل مع 5-(بارا-ن،ن- داي مثيل امينو بنزين)- 2- امينو1,3,4- اوكسودايازولين بوجود كاربونات البوتاسيوم اللامائية ليعطي المركب (A_{30}) ويحضر منه ايضا مركبات الازو وقد تمت دراستها وتشخيصها بواسطة درجات الانصهار وتحاليل اطياف الاشعة تحت الحمراء (FT-IR) كما تم قياس السمية للمركبات المحضرة ومضادات الاكسدة وقابليتها على تثبيط انزيم الزانثين اوكسيديز .

الكلمات المفتاحية: الوبيورينول، مضادات الاكسدة، مركبات الازو، السمية، زانثين اوكسيديز.