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Synthesis and Conformational Analysis of Some Erythropoietin Mimetic Peptides Using Microwave Energy

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THE CURRENT study describes the synthesis of Erythropoietin-which is a prohibited hormone by WADA-mimetic peptides **[**(AFIII54(chain I), (Ser)^{12}AFIII54(chain II), AFIII54 with addition of Gly and Tyr in position 4 and 5, respectively (chain VI), AFIII54 devoid (Thr)^{9} and (Trp)^{10} (chain VII), (EMP3(chain III),(Ser)^{15}EMP3 (chain IV), (Ala)^{15}EMP3(chain V), EMP3 with addition of Gly and Tyr in position 7 and 8, respectively (chain VIII) and EMP3 devoid (Thr)^{12}and (Trp)^{13}(chain IX) **]** by SPPS using microwave energy which accelerated reaction rate and improved the purities and yield.

Conformational analysis using FT-IR spectroscopy showed that most the synthesised peptides existed in α-helix structure**.** Also, biological activity for one of these sequences was studied.

Keywords : Erythropoietin**,** Mimetic peptides**,** Synthesis and Microwave conformational analysis.

Abbreviation: WADA, World Anti-doping Agency; SPPS, solid phase peptide synthesis; Fmoc, 9-fluorenylmethoxycarbonyl. Epo, Erythropoietin; MW, microwave; PS-PEG, polystyrene-divinylbezene-polyethylene glycol graft copolymer; AA, amino acid; HMBA, 4-hydroxymethyl benzoic acid; HOBT, 1-hydroxybenzotriazol; DIC, N, N-diisoprppylcarbodiimide; DMAP, 4-dimethylaminopyridine; AIDS, acquired immune deficiency syndrome; CRF, chronic renal failure.

In the last few years MW-assisted SPPS has been receiving increased attention [1-4] because microwave irradiation mostly accelerated reaction rates and improved the purities and yields in SPPS. Microwave irradiation is an alternative way to conventional heating for providing energy into reactions. The enhancement of solid phase synthesis under microwave irradiation can be explained by both thermal and non thermal microwave effects [5] .One important mechanism in MW-assisted chemical reactions is the dipolar polarization mechanism, which is of particular importance for peptide synthesis .In this mechanism, the alternating electric field from MW radiation provides the energy for the rotation of the molecules having a dipole moment. Unlike conventional heating, MW energy activates any molecule with a dipole moment[6], since the N-terminal amino group and peptide backbone are polar, they constantly try to align with the

alternating electric field of the microwave, and this helps in breaking up the chain aggregation there by improving the coupling efficiency[7]. Also, kinetic of diffusion of reactants from the solution into the resin beads is a key factor of the efficiency of coupling reaction in SPPS[8]. There are several successful publications of microwave-assisted solid phase synthesis of various unnatural biopolymers such as peptoids, pseudo peptides[9], small peptides[10], phosphopeptides[11], difficult peptides[12], peptidyl nucleoside[13], β-peptide libraries[14,15] and glycopeptides[16-18]. In this study nine analogues of erythropoietin mimetic peptides are synthesized because of the importance of Epo as a glycoprotein hormone produced by the kidney. Epo-which is prohibited by WADA- is considered the primary regulator of red blood cell formation in mammals [19].

Material and Methods

The organic solvents and the chemicals used in this part were obtained from Sigma (USA) and Fluka (Switzerland) Chemical Companies. All Fmoc amino acids were purchased from Novabiochem; the used amino acids are of L-configuration. The side chain protecting groups were Pmc for Arg and t-But for Cys, Ser, Thr and Tyr.

Infra-red spectra (KBr) were recorded on FT/IR 1650 perkin-Elmer spectrometer.Amino acid analysis of the peptide sequences were carried out using the amino acid analyzer (Biotronik [LC 6000 E] with an integrator system 1).

Microwave-assisted solid phase peptide synthesis

The synthesis was carried out inside a microwave oven in the nitrogen atmosphere. The reaction vessel (syringe) was located in the middle of the microwave oven (operating at 100W). A Teflon tube from the side arm of the reaction vessel was connected to nitrogen source to introduce a steam of nitrogen during microwave irradiation. The nitrogen gas bubbles served also as a stirrer. The reaction solution was filtered off via the side arm by suction at the end of the reaction.

Coupling of PS-PEG-NH2 with HMBA using MW energy

To a swelled suspension of (0.2g, 1 meq) TentaGel-NH_2 in 4 ml DMF/CH_2Cl_2 (v/ v), a solution of (0.052 g, 4 mmol) HOBt, (0.058 g, 4 mmol) HMBA, (0.484 g, 4mmol) DIC and a catalytic amount of DMAP in 4 ml DMF/CH_2Cl_2 (v/ v) was added. The mixture was then heated in MW oven until Kaiser Test showed a negative result. The polymer was filtered and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether.

Coupling of PS-PEG -HMBA resin to first Fmoc-Gly-OH using MW energy

To a swelled suspension of (0.2 g, cap. = 0.48 meq/ g) HMBA-resin in 4 ml DMF, a solution of (0.114 g, 4mmol) Fmoc-Gly-OH, (0.052 g, 4 mmol) HOBt, (0.0484 g, 4 mmol) DIC and traces of DMAP in 4 ml DMF/ CH_2Cl_2 (v/ v) was added. The mixture was then heated in MW oven for 8 min. The resin was

filtered off and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether.

MW oven power	Time required for complete coupling using conventional method	Time required for complete coupling using MW
100W	12 hr	6 min

Deprotection of Fmoc-N-protecting group using MW energy

FmocA.A.HMBA-resin (0.2g, cap=0.48meq/ g) was suspended in 10ml mixture of 20% piperidine /DMF.The reaction was heated by using MW energy till Kaiser reagent showed +ve result. The resin was filtered off, washed several times by DMF, DCM, DMF, DCM, MeOH and ether then dried.

Coupling of the other Fmoc-A.A. using MW energy

To a swelled suspension of (0.2 g, cap. = 0.48 meq/ g) H-A.A-HMBA-resin in 4ml DMF, a solution of (4 mmol) Fmoc-A.A-OH, (0.052 g, 4 mmol) HOBt, (0.048 g, 4 mmol) DIC and traces of DMAP in 4 ml DMF/ CH_2Cl_2 (v/ v) was added. The mixture was then heated in MW oven. Until Kaiser test showed negative result. The resin was then filtered and washed several times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether.

The used weights for each A.A. are Fmoc-Gly(0.114g), Fmoc-Cys (0.153g), Fmoc-Arg(0.254g), Fmoc-Ile(0.136g), Fmoc-Leu(0.136g), Fmoc-Pro(0.129g), Fmoc-Met(0.143g), Fmoc-Thr(0.153g), Fmoc-Ala(0.119g), Fmoc-Trp(0.164g), Fmoc-Tyr(0.176g), Fmoc-Val(0.130g) and Fmoc-Ser(0.147g).

Results and Discussion

Importance of Epo and its related peptides

In the present work we are interested in erythropoietin which is a hormone produced by the kidney that promotes the formation of red blood cells in the bone marrow. Epo is aglycoprotein has a molecular weight of 34,000 consisting of a 165 amino acids poly peptide chain with three N-and one O-glycosylation sites[20].

The kidney cells that make Epo are specialized and are sensitive to low oxygen levels in the blood. These cells release Epo when the oxygen level is low in the kidney which stimulates the bone marrow to produce more red cells and thereby increase the oxygen-carrying capacity of the blood. Epo is the prime regulator of red blood cell production. Its major functions are to promote the differentiation and development of red blood cells and to initiate the production of haemoglobin. The hormone is widely used in the treatment of blood disorders

characterised by low or defective red blood cell production. At present, Epo is applied clinically in the treatment of anaemia in CRF patients, Acquired Immune Deficiency Syndrome (AIDS) and in cancer patients undergoing chemotherapy [21-23].

Erythropoietin is some times used by athletes to increase their body's oxygen carrying capacity and thus gain an unfair advantage in competition. Besides the risk of disqualification for cheating, athletes who participate in this illicit use of erythropoietin risk the complications of abnormally high red blood cell concentrations, which include abnormal blood clotting so, erythropoietin (Epo) is prohibited according to the World Anti Doping Code [24].

Synthesis of Epo mimetic analogues

The synthesis was carried out by using Fmoc SPPS. 4-Hydroxymethyl-benzoic acid (HMBA) was used as an anchoring group which binds the polymeric support (polystyrene-polyethylene glycol) to the first amino acid. The couplings were mediated by N, N-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazol (HOBT) in N, N-dimethylfomamide (DMF). Deprotection of Fmoc group was carried out using 20% piperidine/DMF mixture. All coupling and deprotection reactions were monitored by Kaiser Test.

The following Epo mimetic peptides analogues which has minimum consensus sequence Tyr-X-Cys-X-X-Gly-Pro-X-Thr-Trp-X-Cys-X-Pro where X represents positions allowing occupation by several amino acids[25] were synthesised using MW energy.

I) Gly-Gly-Cys-Arg-Ile-Gly-Pro-Ile-Thr-Trp-Val-Cys-Gly-Gly
II) Gly-Gly-Cys-Arg-Ile-Gly-Pro-Ile-Thr-Trp-Val-Ser-Gly-Gly
III)Gly-Gly-Val-Tyr-Aal-Cys-Arg-Met-Gly-Pro-Ile-Thr-Trp-Val-Cys-Ser-Pro Leu- Gly-Gly
IV)Gly-Gly-Val-Tyr-Aal-Cys-Arg-Met-Gly-Pro-Ile-Thr-Trp-Val-Ser-Ser-Pro Leu- Gly-Gly
V)Gly-Gly-Val-Tyr-Aal-Cys-Arg-Met-Gly-Pro-Ile-Thr-Trp-Val-Aal-Ser-Pro-Leu- Gly-Gly
VI) Gly-Gly-Cys-Gly-Tyr -Arg -Ile-Gly-Pro-Ile-Thr-Trp-Val-Cys-Gly-Gly
VII) Gly-Gly-Cys-Arg -Ile-Gly-Pro-Ile-Val-Cys-Gly-Gly
VIII)Gly-Gly-Val-Tyr-Aal-Cys-Gly-Tyr-Arg-Met-Gly-Pro-Ile-Thr-Trp-Val-Cys-Ser- Pro -Leu- Gly-Gly
IX) Gly-Gly-Val-Tyr-Ala-Cys-Arg-Met-Gly-Pro-Ile-Val-Cys -Ser-Pro-Leu-Gly-Gly

Then, we cleavage of the peptide (IX) from the resin with alkali to give peptide (X) where the biological activity carried on it.

The time required for complete coupling and deprotection reactions in the MW SPPS of peptide sequences (I-IX) is summarized in Tables 1-9

TABLE 1. Time of complete coupling and deprotection for peptide chain (I).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling/min	Deprotection /min
1	Gly	8	3
2	Gly	4	3
3	Cys	4	3
4	Arg	4	4
5	Ile	3	2
6	Gly	2	1
7	Pro	3	1
8	Ile	3	2
9	Thr	3	2
10	Trp	3	1
11	Val	4	1
12	Cys	4	3
13	Gly	1.5	1
14	Gly	1.5	0.5

TABLE 2. Time of complete coupling and deprotection for peptide chain (II).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling/min	Deprotection /min
12	Ser	2.5	1.5
13	Gly	1.5	1
14	Gly	1.5	0.5

N.B. From amino acid number (1) to amino acid number (11) like that in Table 1.

TABLE 3. Time of complete coupling and deprotection for peptide chain (III).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection /min
1	Gly	8	1.5
2	Gly	2	1
3	Val	4	1
4	Tyr	3	1
5	Ala	2	1
6	Cys	2	3
7	Arg	2	1.5
8	Met	3	1
9	Gly	1	0.5
10	Pro	3	1
11	Ile	3	3
12	Thr	1.5	0.5
13	Trp	2	1
14	Val	3	0.5
15	Cys	3	1
16	Ser	0.5	0.5
17	Pro	2	3
18	Leu	2	1.5
19	Gly	0.5	0.5
20	Gly	0.5	0.5

TABLE 4. Time of complete coupling and deprotection for peptide chain (IV).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection / min
15	Ser	2	1
16	Ser	2	1
17	Pro	3	1
18	Leu	4	2
19	Gly	0.5	0.5
20	Gly	0.5	0.5

N.B. From amino acid number (1) to amino acid number (14) like that in Table 3.

TABLE 5. Time of complete coupling and deprotection for peptide chain (V).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection /min
15	Ala	2	1
16	Ser	2	1
17	Pro	3	1
18	Leu	4	2
19	Gly	0.5	0.5
20	Gly	0.5	0.5

N.B. From amino acid number (1) to amino acid number (14) like that in Table 3.

TABLE 6. Time of complete coupling and deprotection for peptide chain (VI).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection /min
1	Gly	8	0.5
2	Gly	2	0.5
3	Cys	4	3
٤	Gly	٤	٢
5	Tyr	4	3
6	Arg	2	1
7	Ile	1.5	0.75
8	Gly	4	0.5
9	Pro	1.5	1
10	Ile	2.5	3
11	Thr	1.5	1
12	Trp	3	0.75
13	Val	3.5	0.5
14	Cys	2.5	3
15	Gly	2.5	0.5
16	Gly	3	0.5

TABLE 7. Time of complete coupling and deprotection for peptide chain (VII).

A.A NO	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection /min
4	Arg	1.5	2
5	Ile	1.5	1
6	Gly	0.5	0.5
7	Pro	1.5	1
8	Ile	2	0.75
9	Val	4.5	0.5
10	Cys	1	1.5
11	Gly	2	0.5
12	Gly	2.5	0.5

N.B. From amino acid number (1) to amino acid number (3) like that in Table 6.

TABLE 8. Time of complete coupling and deprotection for peptide chain (VIII).

A.A NO.	Fmoc-AA Entry	Time of complete	
		Coupling /min	Deprotection /min
1	Gly	8	1.5
2	Gly	3.5	3
3	Val	4	0.7
4	Tyr	2.5	1
5	Ala	1.5	1
6	Cys	2.5	1
7	Gly	0.75	0.3
8	Tyr	2.5	1
9	Arg	4.5	1.5
10	Met	3.5	0.5
11	Gly	0.7	0.3
12	Pro	2.5	0.7
13	Ile	3.5	6
14	Thr	1.5	0.5
15	Trp	2	0.75
16	Val	4	0.5
17	Cys	2.5	3
18	Ser	1	1
19	Pro	3	8
20	Leu	2	1
21	Gly	0.5	0.3
22	Gly	0.5	0.5

TABLE 9. Time of complete coupling and deprotection for peptide chain (IX).

A.A NO	AA Entry	Time of complete	
		Coupling /min	Deprotection /min
7	Arg	2	1.5
8	Met	4	0.75
9	Gly	0.7	0.3
10	Pro	3	0.7
11	Ile	2	3
12	Val	3.5	0.3
13	Cys	1.5	0.7
14	Ser	0.7	0.75
15	Pro	3	8
16	Leu	2	1
17	Gly	0.5	0.3
18	Gly	0.7	0.5

N.B. From amino acid number (1) to amino acid number (6) like that in Table 8.

The above results indicate clearly that the rates of both coupling and deprotection reactions were markedly enhanced by using microwave energy. Although coupling reactions were carried out by using only four folds excess of amino acids instead of ten folds commonly used. There was a drastic decrease in the time required for complete coupling reactions (2- 4min) compared to (9- 20 hr) in the conventional SPPS. Also, the periods required for completing deprotection reactions decreased to only (0.5-4 min). Compared to (1.5 hr) in the conventional SPPS. Also, it was noticeable that the time required for complete deprotection of Fmoc-Pro was (1min) except (pro) ^{19} in chain (VIII) and (pro) ^{15} in chain (IX) which lasted for (8 min). Also, deprotection of Fmoc-Ile was (3min) except (Ile) ^{13}in peptide chain (VIII) was (6min).These results provided obvious benefits of microwave SPPS in the enhancement of both coupling and deprotection reactions.

The AA analysis of peptide chains (I-IX) are summarized in Table 10.

Spectroscopic study on the synthesized peptide chains

Spectroscopic study on the synthesized peptide chains was carried out by infrared (FTIR) spectroscopy which is particularly useful for probing the secondary structures of proteins. The conformational characteristic IR absorption bands are amide A (3200-3400cm^{-1}), amide I (1600-1750cm^{-1}) and amide V (600-720cm^{-1}). Amide V band is excluded due to the interference of the aromatic ring stretching mode of the resin .The most useful infrared band for the analysis of the secondary structure is the amide I band. Characteristic amide I band assignments of secondary structures are (1695-1670cm^{-1}) band assigned to intermolecular β-structure, (1690-1680cm^{-1}) band assigned to intramolecular β-structure, (1666-1659cm^{-1}) band assigned to '3-turn' helix, (1657-1648cm^{-1}) band assigned to α-helix, (1645-1640cm^{-1}) band assigned to random coil, (1640-1630cm^{-1}) band assigned to intramolecular β-structure and (1625-1610cm^{-1}) band assigned to intermolecular β-structure[(26)].

TABLE 10. Results of A.A. analysis.

Peptide	Amino acids												
I	A. A	G	C	R	I	P	T	W	V				
	Calculated	5	2	1	2	1	1	1	1				
	Found	4.9	1.81	0.82	2	0.87	0.83	0.89	1				
II	A. A	G	C	R	I	P	T	W	V	S			
	Calculated	5	1	1	2	1	1	1	1	1			
	Found	4.95	0.83	0.81	2	0.88	0.81	0.87	1	0.82			
III	A. A	G	V	Y	A	C	R	M	P	I	T	W	S	L
	Calculated	5	2	1	1	2	1	1	2	1	1	1	1	1
	Found	5	2	0 .83	1	1.78	0 .78	0 .79	1.9	1	0.77	0 .78	0.89	
IV	A. A	G	V	Y	A	C	R	M	P	I	T	W	S	L
	Calculated	5	2	1	1	1	1	1	2	1	1	1	2	1
	Found	4.9	2	0.85	1	0.80	77	0.89	1.87	0.98	0.93	0 .87	1.79	0.91
V	A. A	G	V	Y	A	C	R	M	P	I	T	W	S	L
	Calculated	5	2	1	2	1	1	1	2	1	1	1	1	
	Found	4.99	1.95	0 .8	2	0.86	0.86	0.89	1.9	1	0.81	0.89	0.91	0.95
VI	A. A	G	C	Y	R	I	P	T	W	V				
	Calculated	6	2	1	1	2	1	1	1	1				
	Found	5.8	1.77	0 .83	0.81	2	0.89	0.87	0.8	1				
VII	A. A	G	C	R	I	V	P							
	Calculated	5	2	1	2	1	1							
	Found	5	1.8	0.71	1.9	1	0.87							
VIII	A. A	G	V	Y	A	M	R	C	P	I	T	W	L	S
	Calculated	6	2	2	1	1	1	2	2	1	1	1	1	1
	Found	6	1.9	1.78	1	0 .81	0 .8	1.7	1.83	1	0.89	0 .88	1	0.71
IX	A.A	G	V	Y	A	P	M	R	C	I	S	L		
	Calculated	5	2	1	1	2	1	1	2	1	1	1		
	Found	5	1.93	0.82	1	1.88	0 .84	0.78	1.65	1	0 .78	1		

The IR for the synthesized peptides give the following amide I bands. Peptide chain I exhibited a strong band at (1662cm^{-1}) characteristic of '3-turn' helix, on the other hand peptide chains (II, III, V,VI, VII, VIII and X) exhibited a strong band at (1657, 1657, 1645, 1650, 1650 and 1650cm^{-1}), respectively characteristic of α-helix. Also, peptide chains (IV and IX) exhibited a strong band at (1642 and 1645) which assigned to presence of random coil.

The spectroscopic study using IR showed that most of the studied chains are present in α-helix structure except chain (I) which found to be existed in '3-turn' helix and(IV,IX) which exhibited random coil structure.

Biological activity tests

Peptide X was chosen for the biological activity measurements where it was designed to be free of Thr and Trp and it posses an α-helix conformation similar to that of the original Epo according to the conformational study using I.R. spectroscopy.

Thebiological study was carried out using 14 clinically healthy mature adult male albino rats (Ratus ratus), injected I.P. intraperitoneally with synthetic peptide chain X in a dose of 500 I.U/Kg solvated in distilled water. After ten days from injection, individual blood was drawn by orbital puncture from eye plexus for haematological study.

Data of hematological parameters are tabulated in Table 11 and graphically depicted in Fig. 1. All results tabulated as means ± standard errors. The red blood cell count of treated group was significantly higher than that of control group ($p< 0.05$), as well as the white blood cell count of treated group was significantly increased than control group ($p<0.001$). On the other hand, the platelet count of treated group was significantly elevated compared to control group ($p<0.01$), while the hemoglobin concentration of treated group was significantly lower than that of control group ($p<0.001$). In the rest parameters in Table 11 treated group was significantly lower than that of control group (0.001) except McHc is not significant.

TABLE 11. Hematological parameters.

NO.	RBCs count ($x10^6/mm^3$)		WBCs count ($x10^3/mm^3$)		Platelet count ($x10^3/mm^3$)		Haemoglobin concentration(Hbg /100ml)		Haematocrite pcv%		MCV		MCH		MCHC	
	control	treated	control	treated	control	treated	control	treated	control	treated	control	treated	control	treated	control	treated
1	5.49	8.07	4.95	16.70	732	777	15.68	13.20	49.76	43.70	90.64	54.20	2826.	16.40	31.51	30.20
2	5.82	6.49	6.95	22.00	754	1133	13.38	11.50	53.22	35.80	91.44	55.20	22.99	17.70	25.14	32.10
3	5.63	7.23	3.86	12.80	712	997	16.25	12.10	51.27	37.20	91.07	51.50	28.86	16.70	31.69	32.50
4	5.73	6.73	6.56	17.70	640	1042	14.98	11.90	52.87	38.10	92.27	56.60	26.14	17.70	28.33	31.20
5	5.92	5.89	7.32	9.30	698	1022	15.62	10.70	51.24	36.00	86.55	61.10	22.99	18.20	30.48	29.70
6	5.37	4.81	3.63	13.90	780	912	13.61	9.20	52.99	29.60	98.68	61.50	25.34	19.10	25.68	31.10
7	5.58	6.83	7.28	17.30	720	744	16.12	12.20	48.63	40.10	87.15	58.70	28.89	17.90	33.15	30.40
X	5.65	6.58	5.797	15.67	719.43	946.7	15.09	11.54	51.57	37.21	91.11	56.97	26.21	17.67	29.42	31.03
SE	0.07	0.39	0.61	1.54	16.79	54.13	044	0.48	0.57	1.62	1.51	1.39	0.97	0.34	1.18	0.38
T	—	-2.353	—	-5.953	—	-4.01	—	5.43	—	8.28	—	16.63	—	8.27	—	-1.302
P<	—	0.05	—	0.001	—	0.01	—	0.001	—	0.001	—	0.001	—	0.001	—	N.S.

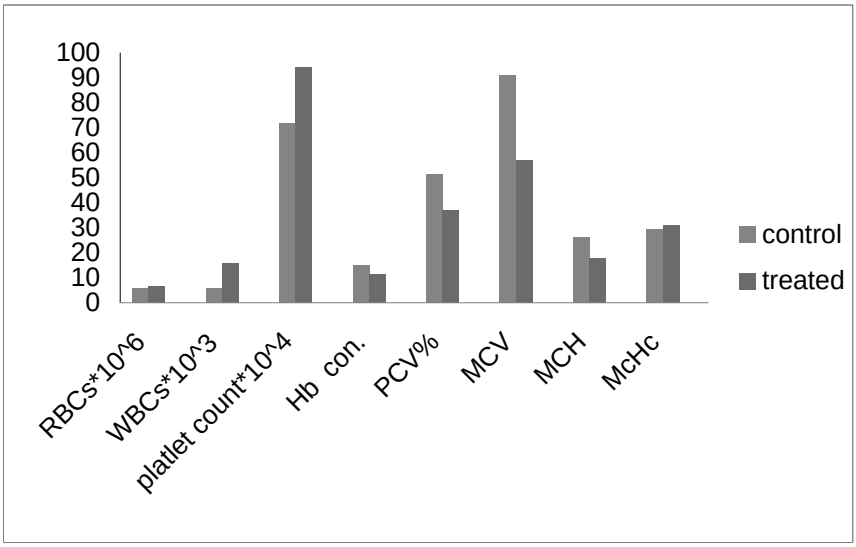

Fig. 1. Histogram of haematopoietic parameters.

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تحضير وتحليل التركيب الفراغي لمركب الإرثروبيوتين وبعض الببتيدات المتشابهة باستخدام طاقة الميكروويف

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تهدف هذه الرساله إلى تحضير بعض متشابهات لهرمون الإرثروبيوتين (الذى يعتبر محرم استخدامه دوليا على الرياضيين من قبل المنظمه الدولية لمكافحة المنشطات) وهذه السلاسل هى:

السلسلة الأولى :(AFIII54) ، والسلسلة الثانية: $(Ser)^{12}$AFIII54 والسلسلة السادسة : AFIII54 مع اضافة Gly و Tyr فى الموضع ٤ و ٥ على التوالى، والسلسلة السابعة : AFIII54 باستبعاد $(Thr)^{9}$ و $(Trp)^{10}$ والسلسلة الثالثة : EMP3 والسلسلة الرابعة : $(Ser)^{15}$EMP3 والسلسلة الخامسة : $(Ala)^{15}$EMP3 والسلسلة الثامنة: EMP3 مع اضافة Gly و Tyr فى الموضع ٧ و ٨ على التوالى، والسلسلة التاسعة: EMP3 باستبعاد $(Thr)^{12}$ و $(Trp)^{13}$ بواسطة طريقة السطح الصلب باستخدام طاقة الميكروويف التى عملت على زيادة سرعة التفاعل وعملت على تحسين النقاوة والناتج.

التحاليل التأكيدية باستخدام التحليل الطيفي FT-IR الذي وضح ان معظم الببتيدات المخلقة توجد على هيئة α-helix. تم عمل دراسة النشاط البيولوجى على احدى هذه السلاسل.