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ORIENTAL JOURNAL OF CHEMISTRY
An International Open Access, Peer Reviewed Research Journal

www.orientjchem.org

ISSN: 0970-020 X
CODEN: OJCHEG
2019, Vol. 35, No.(2):
Pg. 870-876

Synthesis of some New Azo Compounds of Salicylic Acid Derivatives and Determination of their *In vitro* Anti-inflammatory Activity

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http://dx.doi.org/10.13005/ojc/350251

(Received: January 14, 2019; Accepted: April 07, 2019)

ABSTRACT

This study included the preparation of a series of some new azo compounds by diazo coupling aromatic amines with salicylic acid derivatives. The prepared compounds identified using precise elemental analysis (C.H.N.), the results supported the structure of concerned compounds. The synthesized azo compounds also identified by using infrared spectroscopy and 1H-NMR spectroscopy. Anti-inflammatory activity of the compounds were determined in-vitro by human red blood cell (HRBC) membrane stability method, the compounds showed a significant activity to protection of the cell membrane. Other compounds show moderate to low activity, sodium diclofenac was used as positive control.

Keywords: Azo compounds, Salicylic acid, *In vitro* anti-inflammatory and HRBC.

INTRODUCTION

Compounds containing in their structure a group or more of the AZO groups (–N=N–) called azo compounds[1], in which the nitrogen atom hybridization is sp^3 [2]. Because of their physical and chemical properties as well as their biological efficacy, they possess several important applications in pharmaceuticals, cosmetics, textile industry, analytical chemistry and food[1]

Azo compounds are well known to have medical importance, they are recognized in many applications such as antidiabetics[1], and they involved in many biological reactions such as inhibition RNA, DNA, protein synthesis carcinogenesis, and nitrogen fixation[3]. Azo compounds are studied as HIV inhibitors of viral replications[4]. Because of the presence of the azo moiety make these compounds possess biological efficacy as anti-bacterial[5], antitumor[6] insecticide and pesticidal[7,8] activities. Salicylate compounds are widely valued because of their antipyretic, pain killing and anti-inflammation properties. 2-Hydroxybenzoic acid (called also salicylic acid) is most commonly used, and known in salicylates class compounds[9].

The aim of study synthesis of some new azo compounds of salicylic acid derivatives by the diazo coupling reaction and determine their *In-vitro* anti-inflammatory activity by human red blood cell (HRBC) membrane stability method.

EXPERIMENTAL

MATERIAL AND METHODS

p-Nitroaniline, p-aminobenzoic acid, m-nitroaniline, 3-methoxysalicylic acid, 3-methylsalicylic acid and 4-methylsalicylic acid and solvents were used in this study sourced from Sigma-Aldrich company and Merck & Co. The purity of prepared compounds was checked by thin layer chromatography. Melting points recorded by using Gallenkamp apparatus. FT-IR spectra (KBr) of prepared compounds determined on Shimadzu spectrometer (400-4000 cm^{-1}). The proton nuclear magnetic resonance (^{1}H-NMR) spectra determined on Bruker-NMR Spectrometer at 300 MHz using an internal standard $(CH_3)_4Si$ (TMS) and deuterated DMSO-d_6 as a solvent. Elemental analysis and ^{1}H-NMR spectra carried out in Al-albayt University Amman/Jordan.

General method for synthesis of diazonium salts

A solution of an aromatic amine (5 mmol), 1.5 ml of water and 1.5 ml concentrated HCl kept cooled in an ice-salt bath (0°C). A solution of sodium nitrite (5.5 mmol) in 1.5 ml of water added slowly with stirring. The mixture kept at 0°C. for the next step[10,11]. The other diazonium salts synthesized in a similar procedure. Fig. (1) show synthesis of diazonium salt.

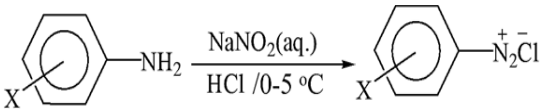

Fig. 1. Synthesis of diazonium salt

Table 1: Dames and physical properties of synthesized azo compounds

Comp.	X	X/	Name	M. p. (°C)	Appearance	Yield (%)
A	3-CH_3	4-NO_2	2-Hydroxy-3-methyl-5-(4-nitro-phenylazo)-benzoic acid	273-275 dec.	Yellow	77
B	3-CH_3	3-NO_2	2-Hydroxy-3-methyl-5-(3-nitro-phenylazo)-benzoic acid	243-245	Light yellow	70
C	3-CH_3	4-COOH	5-(4-Carboxy-phenylazo)-2-hydroxy-3-methyl-benzoic acid	265-267 dec.	Red	83
D	3-OCH_3	4-NO_2	2-Hydroxy-3-methoxy-5-(4-nitro-phenylazo)-benzoic acid	245-247 dec.	Deep red	78
E	3-OCH_3	3-NO_2	2-Hydroxy-3-methoxy-5-(3-nitro-phenylazo)-benzoic acid	236-238	Yellow	89
F	3-OCH_3	4-COOH	5-(4-Carboxy-phenylazo)-2-hydroxy-3-methoxy-benzoic acid	253-255 dec.	Brown	80
G	4-CH_3	4-NO_2	2-Hydroxy-4-methyl-5-(4-nitro-phenylazo)-benzoic acid	235.238 dec.	Orange	91
H	4-CH_3	3-NO_2	2-Hydroxy-4-methyl-5-(3-nitro-phenylazo)-benzoic acid	240-242 dec.	Deep yellow	79
I	4-CH_3	4-COOH	5-(4-Carboxy-phenylazo)-2-hydroxy-4-methyl-benzoic acid	248-250 dec.	Deep red	88

General method for synthesis of azo compounds

The prepared solution of diazonium salt was added portion wise to a solution prepared from salicylic acid derivatives (5.4 mmol) and 10 ml of 2.5 M aq. Sodium hydroxide. The mixture kept with stirring at (0-5°C) for 3-5 hours. The mixture then acidified with conc. HCl (1.5 ml) up to pH ≈ 3. The precipitated compound separated and washed with H_2O. The desired product dried and recrystallized with glacial acetic acid[10,11]. Fig. (2) show synthesis of azo compounds. The melting points, names and the percentage of yield are given in Table (1).

In vitro anti-inflammatory activity[12]

HRBC method was used to estimation *In vitro* anti-inflammatory activity. Blood was collected from healthy volunteers; the blood was mixed with equal volume of sterilized Alsever's solution. The blood solution centrifuged at 3000 rpm and the packed cells were separate. The packed cells were washed with isosaline solution and 10% v/v suspension was prepared by complete the volume with isosaline. Alsever's solution were prepared of 2.05% glucose, 0.42% NaCl, 0.8% trisodium citrate, 0.055% citric acid, all dissolved in water. This solution was using for storage RBC. Other solution were using in this method Hyposaline (0.7% NaCl), Isosaline (0.9% NaCl), phosphate buffer (pH 7.4) and ethanol.

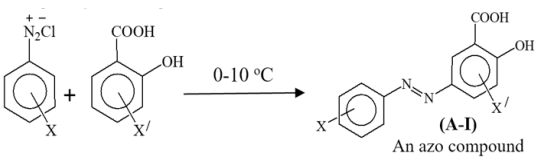

Fig. 2. Synthesis of azo compounds

Tested compounds (75 mg) was dissolve in 1 ml of ethanol. Samples of each compound, control and sodium diclofenac were separately mixed with (1 ml) phosphate buffer, (2 ml) of hyposaline and (0.5 ml) of HRBC suspension. All the assay mixtures were incubate at 36.5^{O}C for 30 min and centrifuged at 3000 rpm for 10 minutes. The supernatant liquid was decanted and haemoglobin content was estimate by spectrophotometer at 560 nm. The percentage of haemolysis protection was estimate by assuming the haemolysis produced in the control as 100%, according to following equation.

Percentage protection=100-(Ac-As/Ac)

Were Ac= Absorption of control and As= Absorption of sample

Statistical analysis

The results of anti-inflammatory activity were analysis in one way analysis of variance (ANOVA). Value with probability ($p<0.01$) was considered significant.

RESULTS AND DISCUSSION

Table (2) show CHN analysis of synthesized azo compounds (A-I) and the practical results support the structure of synthesized compounds.

Table 2: CHN analysis of azo compounds

Compd.	Mol. formula	Mol. weight	Elemental analysis					
			C%		H%		N%	
			Cal.	Found	Cal.	Found	Cal.	Found
A	$C_{14}H_{11}N_3O_5$	301.25	55.82	55.53	3.68	3.90	13.95	13.57
B	$C_{14}H_{11}N_3O_5$	301.25	55.82	55.90	3.68	3.63	13.95	14.20
C	$C_{15}H_{12}N_2O_5$	300.27	60.00	60.17	4.03	4.14	9.33	9.62
D	$C_{14}H_{11}N_3O_6$	317.25	53.00	52.81	3.49	3.57	13.24	13.35
E	$C_{14}H_{11}N_3O_6$	317.25	53.00	52.75	3.49	3.41	13.24	12.51
F	$C_{15}H_{12}N_2O_6$	316.27	56.96	57.13	3.82	4.02	8.86	9.10
G	$C_{14}H_{11}N_3O_5$	301.25	55.82	55.61	3.68	3.45	13.95	13.68
H	$C_{14}H_{11}N_3O_5$	301.25	55.82	56.03	3.68	3.51	13.95	13.73
I	$C_{15}H_{12}N_2O_5$	300.27	60.00	59.83	4.03	3.85	9.33	9.49

FT-IR spectra

The FT-IR spectra (KBr disc) of prepared compound show a strong absorption at (1654-1693 cm^{-1}) for carbonyl carboxylic group[1,13]. All compounds exhibit absorption bands at ranges (1604-1616 cm^{-1}), (1523-1577 cm^{-1}) and (1438-1477 cm^{-1}) for the stretching vibrations of -N=N- and C=C groups respectively because are superimposed in the same ranges[1,13]. The spectra of the azo compounds show strong absorption bands at ranges (1273-1288 cm^{-1}), (1195-1222 cm^{-1}), (1249-1265 cm^{-1}) and (1300-1354 cm^{-1}) due to the stretching vibrations for (C-O, carboxylic), (C-O, phenolic), (C-N) and (NO_2) respectively[1,13,14]. The other FT-IR vibrations of the synthesized compounds are shown in Table (3).

^{1}H-NMR spectra

The proton-NMR analysis of prepared compounds performed by using deuterated dimethyl sulfoxide as a solvent. The ^{1}H-NMR spectra of all prepared azo compounds showed a singlet signal within the ranges (2.264-2.296 ppm), (3.918-3.931 ppm) and (2.681-2.725 ppm) for the groups 3-CH_3, 3-OCH_3 and 4-CH_3 respectively. Compounds A, B and C showed a singlet signals at (7.785-8.042 ppm) and (8.132-8.507 ppm) which attributed to protons H-4 and H-6 respectively. The ^{1}H-NMR spectra of the synthesized compounds for compounds D, E and F showed singlet signals at ranges (7.675-7.732 ppm) and (8.050-8.444 ppm) for protons H-4 and H-6 respectively with 4J= 2.1. In addition to that compounds G, H and I appeared singlet signals at (7.013-7.072 ppm). Proton (H-6) for compounds G and H appeared at 8.171 ppm and 8.175 ppm respectively[15-17], the general structure of azo compounds was shown in Fig. (3). Other aromatic protons of all azo synthesized compounds summarized in Table (4). The data of ^{1}H-NMR spectra of synthesized compounds reported in Figures (4 – 12).

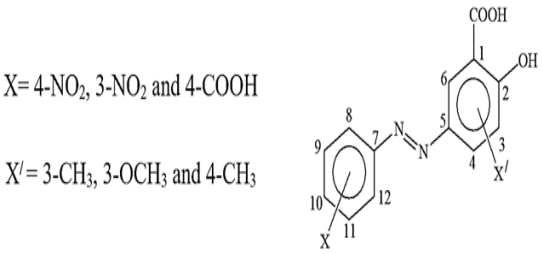

Fig. 3. General structure of azo compounds

Table 3: Data of FT-IR spectra (cm^{-1}) of synthesized azo compounds

Compd.	C=O	N=N C=C	C=C	C-O carboxylic str.	C-O phenolic str	C-N str.	C-H Aromatic str,	C-H Aromatic bend.	NO_2 (Str.) sym.	O-H Carboxylic	O-H phenolic	C-H aliphatic str.
A	1654	1527	1608	1276	1222	1257	3039	852	1346	2519	3417	2854
		1438					3078	910		2600	3587	2989
B	1658	1531	1612	1284	1222	1257	3028	848	1354	2525	3417	2870
		1446					3082	910		2603	3475	2955
C	1654	1577	1604	1288	1222	1265	3066	867	1300	2596	3414	2866
	1685	1446					3160	906		2661	3502	2989
D	1678	1580	1609	1261	1230	1261	3082	887	1342	2645	3313	2997
		1458						914				
E	1667	1585	1613	1285	1199	1267	3093	879	1350	2603	3421	2870
		1469					3194	898		2754	3522	2993
F	1658	1523	1608	1276	1223	1277	3035	860	1320	2620	3522	2854
	1680	1454					3100	910		2730	3595	2962
G	1666	1527	1608	1273	1219	1253	3074	794	1342	3074	3479	2858
		1442					3160	850		3159	3552	2927
H	1685	1570	1616	1276	1211	1249	3101	864	1346	2640	3414	2840
		1477						894			3482	2927
I	1670	1581	1604	1284	1195	1250	3090	860	1342	2540	3479	2860
	1693	1485						910		2700	3552	2910

Str. = stretching, bend. = bending, sym. = symmetrical

Table 4: Data ^{1}H-NMR for compounds A to I in DMSO-d_6

		^{1}H-NMR, δ (ppm), ^{n}J H-H (Hz)	
Comp.	X'	C-H (aliphatic)	(C-H) aromatic
A	3-CH_3	2.264 (3H)	7.785(H-4), 8.232(H-6), 7.952, (d, H-8, H-12, $^3J_{8,9}$,$^3J_{12,10}$= 8.7), 8.371(d, H-9, H-11, $^3J_{9,8}$= $^3J_{11,12}$= 8.7)
B	3-CH_3	2.296 (3H)	8.042(H-4), 8.507(H-6), 7.876(t, H-9, $^3J_{9,8}$= $^3J_{9,10}$= 8.1), 8.266-8.373(m, H-8,H-10,H-12)
C	3-CH_3	2.294 (3H)	8.005(H-4), 8.132(H-6), 7.919(d, H-8, H-12, $^3J_{8,9}$=$J_{12,11}$= 8.4), 8.118(d, H-9, H-11, $^3J_{9,8}$= $^3J_{11,12}$= 8.4)
D	3-OCH_3	3.918 (3H)	7.675(d,H-4, ^{4}J= 2.1), 8.063(d, H-6, ^{4}J= 2.1), 8.045(d, H-8, H-12, $^3J_{8,9}$= $^3J_{12,11}$= 9), 8.409(d, H-9, H-11, $^3J_{9,8}$= $^3J_{11,12}$= 9)
E	3-OCH_3	3.931 (3H)	7.732(d,H-4, ^{4}J= 2.1), 8.444(d,H-6, ^{4}J= 2.1), 7.890 (t, $^3J_{9,8}$= $^3J_{9,10}$= 8.1), 8324-8.392(m, H-8, H-10), 8.650(d, H-12, ^{4}J= 1.8)
F	3-OCH_3	3.923 (3H)	7.690(d, H-4, ^{4}J= 2.1), 8.050(d, H-6, ^{4}J=2.1), 7.952(d, H-8, H-12, $^3J_{8,9}$= $^3J_{12,11}$= 8.7), 8.130(d, H-9, H-11, $^3J_{9,8}$= $^3J_{11,12}$= 8.7)
G	4-CH_3	2.725 (3H)	7.072(H-3), 8.171(H-6), 8.066(d, H-8, H-12, $^3J_{8,9}$= $^3J_{12,11}$= 9), 8.412(d, H-9, H-11, $^3J_{9,8}$= $^3J_{11,12}$= 9)
H	4-CH_3	2.725 (3H)	7.066(H-3), 8.175(H-6), 7.880(t, H-9, $^3J_{9,8}$=$^3J_{9,10}$= 8.1) 8.322-8.381(m, H-8, H-10), 8.532(H-12)
I	4-CH_3	2.681 (3H)	7.013(H-3), 7.913(d, H-8, H-12, $^3J_{8,9}$, $^3J_{12,11}$=7.5)8.106 (3H, H-6, H-9, H-11)

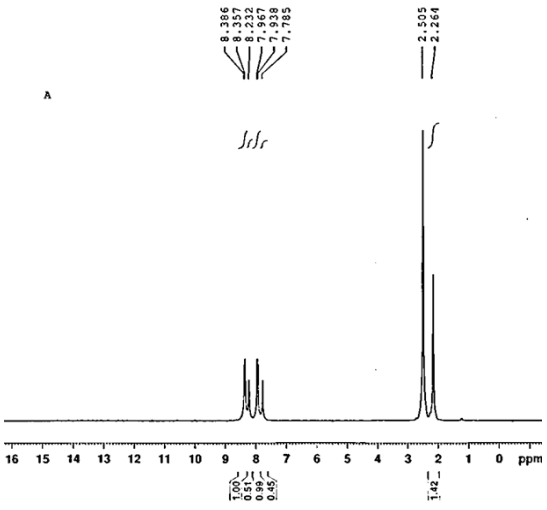

Fig. 4. ^{1}H-NMR spectrum of azo compound A

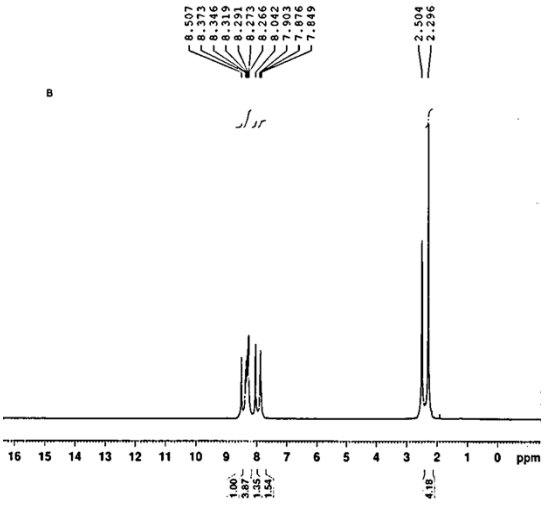

Fig. 5. ^{1}H-NMR spectrum of azo compound B

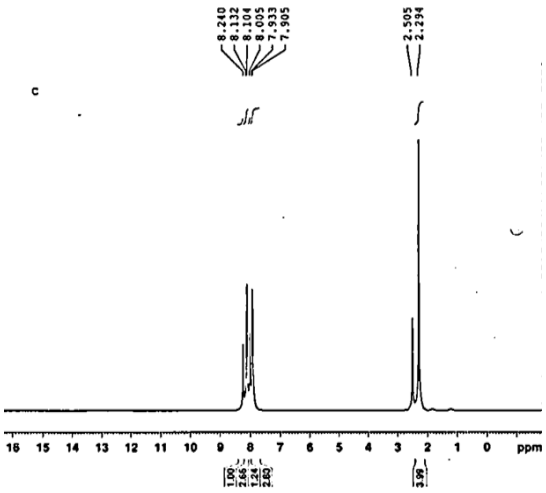

Fig. 6. ^{1}H-NMR spectrum of azo compound C

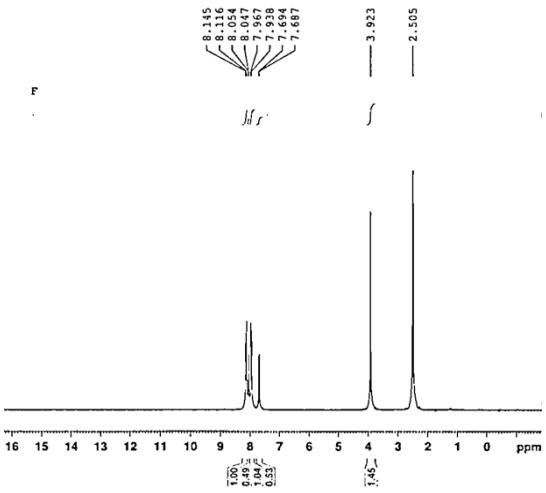

Fig. 9. ^{1}H-NMR spectrum of azo compound F

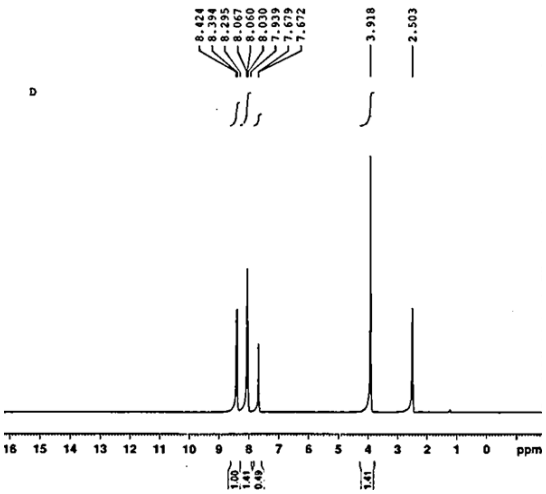

Fig. 7. ^{1}H-NMR spectrum of azo compound D

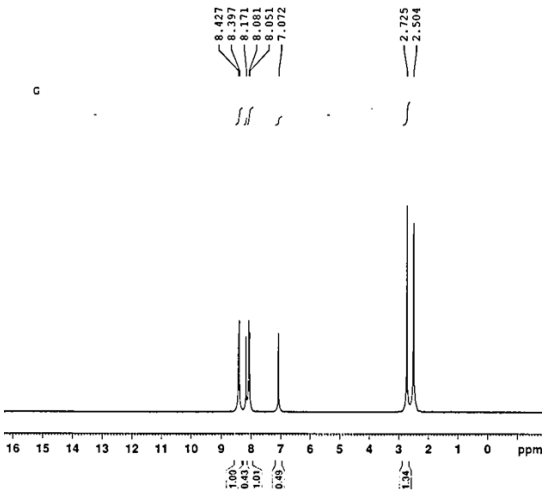

Fig. 10. ^{1}H-NMR of azo compound G

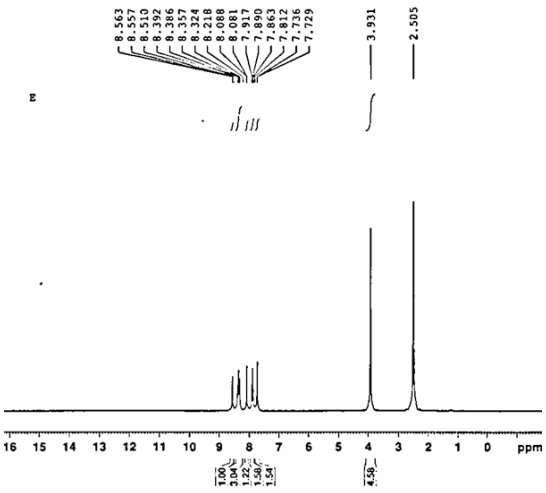

Fig. 8. ^{1}H-NMR of azo compound E

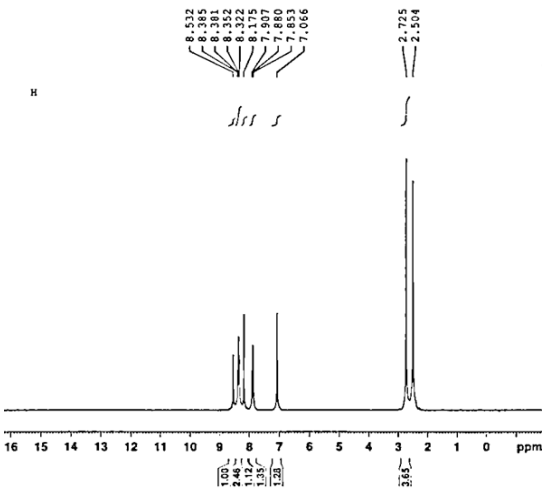

Fig. 11. ^{1}H-NMR of azo compound H

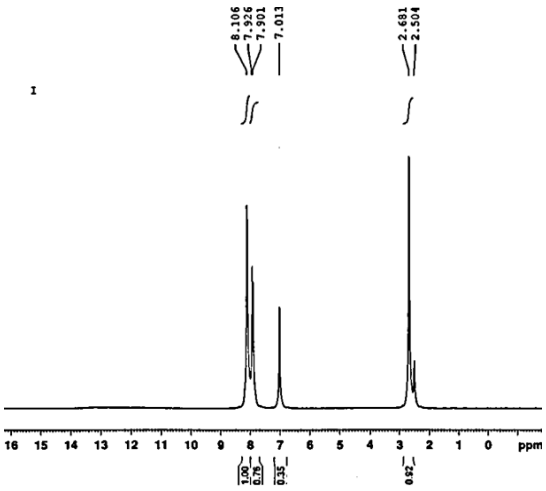

Fig. 12. ^{1}H-NMR of azo compound I

***In vitro* anti-inflammatory activity**

In inflammation, there is extra cellular fluid release because cell damage, lysosome preventing the inflammation response if lysosomal membrane still stable. Lysosomal contain active enzyme like bactericidal enzyme and proteases, this cause additional tissue injury. So stability of lysosomal membrane very important for inflammatory prevention. The HRBCs membrane are used as analogous to lysosomal membrane, because they have similar components, a protection of HBRCs membrane of lysis were rated as anti-inflammatory activity[18].

All the synthesized azo compounds (A-I) showed a significant anti-inflammatory activity by HRBC membrane stabilization method. The results of the activity are listed in Table (5).

Table 5: Protection of HRBC membrane of compounds at dose 75mg

The compound	Protection %
A	20.16
B	69.53*
C	31.86
D	28.30
E	71.87*
F	25.15
G	23.95
H	79.76*
I	30.43
Sodium diclofenac	74.48*

*= p< 0.01

From the results, compounds B, E and H show high activity compared with other compounds. The active compounds B, E, and H contain a nitro group at meta position. Replacing nitro group with carboxyl group will be minimize the activity. Therefore, the activity is the best when a nitro group located at meta position. In salicylic acid moiety, the activity of protection was found increased when methyl group located at para-position compared with the meta-methyl and meta-methoxy groups. Compound E contain meta nitro group and meta methoxy group, it is the compound with meta-methoxy group has high activity than other methoxy derivatives. The results showed compounds A, D, F and I have less activity. The action of the azo compounds could be related to the binding of compounds with erythrocyte membrane, especially phospholipids. Since the compounds has polar and nonpolar groups can bonded with same groups of phospholipids. This prevents membrane damage by physical interaction of osmotic pressure differences, which is causative to hemolysis of red blood cells[19].

CONCLUSION

In this study, three series of azo were prepared using three different of salicylic acid derivatives. The prepared compounds identified using the element analysis (CHN) as well as infrared and ^{1}H-NMR spectroscopy. The results supported the structures of prepared compounds. All compounds showed significant *In vitro* anti-inflammatory activity. Compounds B, E and H showed high anti-inflammatory activity compared with other prepared azo compounds.

ACKNOWLEDGMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors have no conflict of interest to publish the article.

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