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Synthesis, characterization, antioxidant power and acute toxicity of some new azo-benzamide and azo-imidazolone derivatives with in vivo and in vitro antimicrobial evaluation

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ABSTRACT

In this research paper, a stepwise chemical reaction was conducted to synthesize and develop of a new potent azo-oxazolone, which was used as prototypical molecule for production of two series of azo-benzimide (5a–j) and azo-imidazolone (6a–j). FT-IR, ^{1}H NMR, ^{13}C NMR and CHN analysis were used for the structural elucidation. The high biological efficiency of newly obtained compounds was confirmed by in vitro antioxidant efficacy and in vitro antimicrobial activity against gram-positive and gram-negative bacteria via disc diffusion and tube dilution techniques. In addition, in vivo anti-microbial activity of some of the synthesized compounds was determined by using burnt rats which infected by *Staphylococcus aureus*. Tested compounds have shown high anti-microbial activity and wound healing in comparison to ucederm as a control. In vivo acute toxicity was carried out by up and down method for the compounds 4, 5d and 6d. The limited test dose was 2000 mg/kg, while the maximum tolerated dose was 5000 mg/kg which has administered no lethality recorded.

1. Introduction

The 5-(4*H*)-oxazolones or Erlenmeyer azlactones are five membered heterocyclic compounds conveniently accessible by condensation of substituted hippuric acids with the corresponding substituted benzaldehydes in the presence of acetic anhydride and sodium acetate [1,2]. In terms of the reactivity, oxazolone **4** resemble lactone, and therefore react with diverse nucleophiles via ring opening as the initial step of their conversion. 5-(4*H*)-oxazolones are serving as privilege structures in the synthesis of various important compounds such as imidazolones [3], benzamides [4], non-natural amino acids [5], peptides [6] and pericyclic cycloaddition reaction [7].

5-(4*H*)-Imidazolone and its derivatives have widely been used in drug discovery particularly for antitumor [8], anti-cancer [9,10], antiproliferative [11], anti-inflammatory [12], antihypertensive [13] and antiepileptic [14] diseases. Moreover, due to the long π-conjugated electrons their optical properties of imidazolones could lead to potential applications in micro fabrication, such as optical imaging [15] and for preparation of green fluorescent protein (GFP) [16,17].

In the past decades, a number of published studies show the degradation and toxicity of the azo dyes in both aerobic and anaerobic conditions [18–20]. In contrast to these disadvantages, there are several studies showing the low-toxicity and stability of these dyes for long-term (72 days) incubations [21]. More specifically some oxazolones, benzamides and imidazolones containing azo linkage have been shown to exhibit a broad range of pharmacological and biological activities, including antimicrobial activity [22] and tyrosinase inhibition activity [23].

A variety of animal models over the last decade have focused on applying well-known treatments for traditional mechanisms of burn wound progression [24,25]. Wounds and especially infected wounds caused by *S. aureus* have posed an increasingly severe threat to human's health all over the world [26]. Thus, during the wound repairing progress, infection control, healing promotion and defined biological safety limits are equally important of these antimicrobial materials to promote the healing of the infected wound.

In Recent decades, literature survey showed that many researchers in worldwide focused on oxazolones and imidazolones for drug discovery [27-29], but very few of them focused on azo-oxazolones and azo-imidazolones [22,23,30,31]. Herein, In the light of all these facts, this work deals with the synthesis of an azo-oxazolone and successfully modified the desired addition mode of either an efficient fast nucleophilic ring opening of **4** by various substituted anilines to **(5a–j)** followed by intramolecular cyclodehydration in acidic solution to afford azo-imidazolone derivatives **(6a–j)** in excellent yields. Followed by in vivo and vitro antimicrobial activities and acute toxicity for some of the

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newly produced compounds as an ideal nontoxic material for infected wound repairing and as a preliminary studies for future engineering in the course of our drug discovery programs.

2. Methodology

2.1. Chemistry

All the chemicals used in Scheme 1 were of analytical grade and they were obtained from different commercial sources (Fluka, BDH, and Riedel-de Haen) and used without any further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker spectrometer (400 MHz) with $CDCl_3$ as a solvent at Jordan university of science and technology. Chemical shifts are expressed in parts per million (δ) using residual solvent protons as internal standard (δ 7.26 in ^{1}H and δ 77.0 in ^{13}C). Splitting patterns are designated as *s* (singlet), *d* (doublet), *t* (triplet), *m* (multiplet). Elemental analyses were carried out with VarioMicro V3.1.1 CHNS analyzer at University technology of Malaysia. Infrared spectra were recorded with IRaffinity-1 (Shimadzu) spectrometers with KBr pellets. The spectral records were performed on a Shimadzu UV-1800 Series single beam UV/Vis scanning spectrophotometer. Melting points were determined by using Electro thermal melting point apparatus model 9100 (capillary method) and are uncorrected.

2.1.1. Preparation of 4-Bromohippuric acid (1)

According to previously published procedure [32], a solution of glycine (8.25gm, 0.11Mol) and potassium hydroxide (0.2Mol, in 50 mL) was cooled to 0 °C then 4-Bromobenzoylchlororide (21.95gm, 0.1Mol) was added in two portions with shaking. Further, the reaction mixture was stirred for 30 min. After that, the solution was acidified with conc. HCl and the product was collected (25gm) and recrystallized from ethanol.

Yield: 96.89%, **M.P:** found (155–157) °C, IR. **(KBr)** (ν_{max}, Cm^{-1}) 3296 (NH), 2550–3296 (acid OH), 1703 (acid C=O), 1635 (amide C=O).

2.1.2. Preparation of 5-((3-methylphenyl)diazenyl)-2-hydroxybenzaldehyde (2)

The purified compound (2) was prepared according to our developed procedure [33] in an excellent yield. The solid product dried and recrystallized from a mixture of toluene and ethanol (1:4) to give (brown-yellow) crystals of compound **(2).**

$C_{14}H_{12}N_2O_2$; **M.P:** 160–161 °C; **yield**: 91%, **IR(ν_{max}/cm^{-1}):** 3186, 2839, 2733, 1660, 1579, 1479, 1280.

2.1.3. Preparation of (E)-2-(benzyloxy)-5-((3-methylphenyl)diazenyl) benzaldehyde (3)

According to described procedure [34], a solution of benzylbromide (13.68gm, 0.08 mol) and 5-((3-methylphenyl)diazenyl)-2-hydroxybenzaldehyde (12gm, 0.05Mol) in (100 mL) of ethanol 96% were refluxed for 10 min. Anhydrous Na_2CO_3 (15.9gm, 0.15Mol) was added in three portions to the reaction mixture. Further refluxed continued for six hours, then the reaction mixture was added to cold distilled water (150 mL) to give an orange precipitate, which was filtered off and washed with a mixture of cold water and ethanol (1:1) to remove excess of salts and benzyl bromide, then recrystallized from a mixture of toluene and ethanol (1:4) to give an orange-brownish precipitate **(3).**

$C_{21}H_{18}N_2O_2$ Orange solid, **yield:** 15.5gm (93.9%); **M.P:** 86–88; **IR (ν_{max}/cm^{-1}):** 2864, 2765, 1683(C=O), 1602 (C=C ring), 1577(N=N), 1253–1161(C–O–C). ^{1}H NMR **(δ, ppm):** 2.27–2.55 (s, 3H–Ar–CH_3), 5.25 (s, 2H–OCH_2), 7.12–8.42 (m, 12H–Ar–H), 10.57 (s, 1H– aldehydic group). ^{13}C NMR **(δ, ppm):** (21.44:C–Ar–CH_3, 71.32:C–OCH_2, 113.52:C_3, 120.52:C_{12}, 123.66:C_8, 123.68:C_6, 125.49:$C_{14,18}$, 127.43:C_{16}, 128.56:C_{10}, 128.90:C_1, 128.99:$C_{15,17}$, 129.76:C_{11}, 131.88:C_4, 135.66:C_{13}, 139.06:C_9, 146.74:C_5, 152.63:C_7, 162.60:C_2, 189.24:C=O.

2.1.4. Synthesis of an azo-oxazolone; 4-((Z)-2-(benzyloxy)-5-((E)-m-tolyldiazenyl)benzylidene)-2-(4-bromophenyl)oxazol-5(4H)-one (4):-

According to modified procedure [35], (6.6gm, 0.020Mol) of compound **3** and glacial acetic acid (15 mL) stirred under reflux. Then freshly fused sodium acetate (2.46gm, 0.030Mol), 4-bromohippuric acid (5.42gm, 0.021Mol) and acetic anhydride (15 mL) were added. The reaction mixture immediately became soluble, after 2 min. Orange crystals appeared in the solution, during 5 min large quantity of precipitate of azo-oxazolone formed and the reaction mixture was refluxed for further 5 min., *(herein, during the reaction the volume of acetic acid and time very important because, in the presence excess of acetic acid and extra time, the product become soluble)* the mixture was transferred in to a beaker and the flask rinsed with 20 mL ethanol then 15 mL of water added dropwise with stirring, then the reaction mixture left in refrigerator for 30 min. The orange product was collected and washed with cold aqueous sodium carbonate (50 mL, 4%) to afford the desired compounds **(4)**.

$C_{30}H_{22}BrN_3O_3$ Orange solid, **yield:** 10.5gm (95%), **M.P:** 180–181.2 °C. **IR (ν_{max}/cm^{-1}):** 1797(C=O) with shoulder at 1766, 1649 (C=N), 1587(C=C), 1552 (N=N), 1481, 1253, 1159. ^{1}H NMR **(δ, ppm):** 2.41 (s, 3H, Ar-CH_3), 5.19 (s, 2H, –OCH_2), 7.00–7.99 (m, 15H, Ar–H), 9.4 (s, 1H, vinylic H). ^{13}C NMR 21.55:C Ar–CH_3, 70.96:C–OCH_2–, 112.51:C_3, 120.38:C=C, 123.12:C_1, 123.29:C_6, 124.60:C_{12}, 125.43:C_8, 127.09:C_4, 127.38:C_{19}, 128.33:C_{22}, 128.38:$C_{14,18}$, 128.44:C_{16}, 128.92:C_{10}, 129.09:$C_{15,17}$, 129.77:C_{11}, 131.72:$C_{20,24}$, 132.35:C=C ring, 133.23:$C_{21,23}$, 135.86:C_{13}, 139.05:C_9, 146.94:C_5, 152.68:C_7, 160.15:C=N, 162.74:C_2, 167.22:C=O. **Elemental Anal**. calcd for $C_{30}H_{22}BrN_3O_3$: C, 65.23; H, 4.01; N, 7.61; found: C, 65.19; H, 3.98; N, 7.55.

2.1.5. Synthesis of azo benzamide derivatives N-(-1-(2-(benzyloxy)-5-(m-tolyldiazenyl)phenyl)-3-((substitutedphenyl)-amino)-3-oxoprop-1-en-2-yl)-4-bromobenzamide (5a–j)

In accordance with a previously established method [36] with some modifications, a mixture of an Azo-oxazolone (0.83gm, 0.0015Mol) and 5 mL of acetic acid were heated in a beaker to boil, then the mixture transferred from the heating source to on a sensitive balance (in fuming hood) and (0.0025Mol) of substituted aromatic amine was added drop wise with stirring. Immediately the insoluble orange mixture became liquor solution (in case **5i** and **5j** were refluxed for 30 min), on cooling yellow-orange crystals of **(5a–j)** appeared in the solution then 10 mL of D.W added. The described product was filtered off, washed by D.W, dried and crystallized (twice) in a mixture of toluene and ethanol in which each compound was dissolved in 10 mL of toluene, filtered (hot) and evaporated to half. After cooling ethanol was added drop wise until nice crystals produced (see Table 1).

Table 1
Some physical properties and IR data for the synthesized azo-benzamides (**5a–j**).

Prod.	R	Molecular formula	M.P	%Yield	IR str.cm^{-1}	
					C=O	N–H
5a	H	$C_{36}H_{29}BrN_4O_3$	211–212	83	1645	3240
5b	2-CH_3	$C_{37}H_{31}BrN_4O_3$	192–193	91	1647	3240
5c	3-CH_3	$C_{37}H_{31}BrN_4O_3$	168–169	95	1672, 1658	3388
5d	4-CH_3	$C_{37}H_{31}BrN_4O_3$	210–212	96	1641	3219
5e	2-OCH_3	$C_{37}H_{31}BrN_4O_4$	188–189	92	1670	3244
5f	4-OCH_3	$C_{37}H_{31}BrN_4O_4$	196–197	98	1641	3224
5 g	2-OCH_2CH_3	$C_{38}H_{33}BrN_4O_4$	186–187	82	1662	3230
5 h	4-OCH_2CH_3	$C_{38}H_{33}BrN_4O_4$	316–317	97	1645	3240
5i	3-Cl-4-CH_3	$C_{37}H_{30}BrClN_4O_3$	308–309	98	1641	3263
5j	5-CH_3-py	$C_{36}H_{30}BrN_5O_3$	175–176	78	1678	3236

2.1.5d. N-((Z)-1-(2-(benzyloxy)-5-((E)-m-tolyldiazenyl)phenyl)-3-oxo-3-(p-tolylamino)prop-1-en-2-yl)-4-bromobenzamide ^{1}H NMR (δ, ppm)

2.2 (s, 3H, *p*-CH_3), 2.33–0.246 (s, 3H, *m*-CH_3), 4.74 (s, 2H, −OCH_2), 6.60 (s, 1H, NH-1), 6.84 (s, 1H, NH-2), 7.11–7.94 (m, 19H, Ar−H), 9.24 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):** 20.90:C of p-CH_3, 21.39:C Ar−CH_3, 70.38:C−OCH_2−, 112.91:C_3, 119.68:C_1, 121.08:C_6, 121.45:C_{12}, 122.73:$C_{26,26'}$, 123.14:C_{17}, 123.60:C_8, 125.91:C_4, 126.50:$C_{14,14'}$, 126.88:C_{10}, 127.36:C_{23}, 128.31:C_{16}, 128.49:C_{18}, 128.86:$C_{15,15'}$, 129.27:C_{11}, 129.37:$C_{27,27'}$, 131.65:$C_{21,21'}$, 132.06:$C_{22,22'}$, 132.39:C_{20}, 135.57:C_{25}, 135.76:C_{13}, 136.16:C_{28}, 138.94:C_9, 146.79:C_5, 152.45:C_7, 157.69:C_2, 163.97:C_{19}(C═O), 165.77:C_{24} (C═O). **Elemental Anal.** Calcd. for $C_{37}H_{31}BrN_4O_3$: C, 67.38; H, 4.74; N, 8.49; found: C, 67.22; H, 4.66; N, 8.47.

2.1.5f. N-((Z)-1-(2-(benzyloxy)-5-((E)-m-tolyldiazenyl)phenyl)-3-((4-ethoxyphenyl) amino)-3-oxoprop-1-en-2-yl)-4-bromobenzamide ^{1}H NMR (δ, ppm)

2.39 (s, 3H, *m*-CH_3), 3.70 (s, 3H OCH_3), 4.83 (s, 2H, −OCH_2), 6.64 (s,1H, NH-1), 6.70 (s,1H, NH-2), 7.10–7.88 (m, 19H, Ar−H), 9.07 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):** 21.43:C Ar−CH_3, 55.47:OCH_3, 71.53:C−OCH_2−, 113.61:C_3, 113.89:$C_{27,27'}$, 119.58:C_1, 121.29:C_6, 121.72:C_{12}, 123.10:$C_{26,26'}$, 123.25:C_{17}, 123.64:C_8, 126.49:C_4, 126.85:$C_{14,14'}$, 127.26:C_{10}, 127.41:C_{23}, 128.46:C_{16}, 128.72:C_{18}, 128.84:$C_{15,15'}$, 129.47:C_{11}, 131.55:$C_{21,21'}$, 131.63:C_{25}, 131.70:$C_{22,22'}$, 132.15:C_{20}, 135.79:C_{13}, 138.98:C_9, 146.69:C_5, 152.43:C_7, 156.26:C_{28}, 157.62:C_2, 164.07:C_{19}(C═O), 165.90:C_{24} (C═O). **Elemental Anal.** Calcd. for $C_{37}H_{31}BrN_4O_4$: C, 65.78; H, 4.63 N, 8.29; found: C, 65.45; H, 4.62; N, 8.27.

2.1.5h. N-((Z)-1-(2-(benzyloxy)-5-((E)-m-tolyldiazenyl)phenyl)-3-((4-ethoxyphenyl) amino)-3-oxoprop-1-en-2-yl)-4-bromobenzamide ^{1}H NMR (δ, ppm)

1.24–1.41 (t, 3H, CH_3 of −OCH_2CH_3), 2.34–2.46 (s, 3H, *m*-CH_3), 3.83–3.86 (q, 2H, OCH_2 of −OCH_2CH_3), 4.69 (s, 2H, −OCH_2), 6.54 (s,1H, NH-1), 6.56 (s,1H, NH-2), 7.08–7.91 (m, 19H, Ar−H), 9.37 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):**14.14:CH_3 of OCH_2CH_3, 21.45:C Ar-CH_3, 63.60:CH_2 of OCH_2CH_3, 71.03:C−OCH_2, 113.13:C_3, 114.36:$C_{27,27'}$, 119.36:C_1, 121.37:C_6, 121.54:C_{12}, 122.60:$C_{26,26'}$, 123.38:C_{17}, 123.93:C_8, 126.71:C_4, 126.85:$C_{14,14'}$, 126.99:C_{10}, 127.21:C_{23}, 128.19:C_{16}, 128.35:C_{18}, 128.71:$C_{15,15'}$, 128.86:C_{11}, 129.60:$C_{21,21'}$, 131.43:C_{25}, 131.54:$C_{22,22'}$, 132.82:C_{20}, 135.96:C_{13}, 138.92:C_9, 146.41:C_5, 152.34:C_7, 155.40:C_{28}, 157.78:C_2, 164.50:C_{19}(C═O), 165.99:C_{24} (C═O). **Elemental Anal.** Calcd. for $C_{38}H_{33}BrN_4O_4$: C, 66.18; H, 4.82; N, 8.12; found: C, 65.81; H, 4.45; N, 7.98.

2.1.6. Synthesis of azo-imidazolone 5-((Z)-2-(benzyloxy)-5-((E)-m-tolyldiazenyl)benzylidene)-2-(4-bromophenyl)-3-(2-ethylphenyl)-3,5-dihydro-4H-imidazol-4-one (6a–j):

Azo-imidazolone derivatives **(6a–j)** were synthesized as reported in procedure [37] by mixing of azo-oxazolone (0.83gm, 0.0015Mol) with a substituted aromatic amine (0.003Mol) in acetic acid (10 mL) and refluxed with stirring for (2–15) h. The reaction mixture was poured into water and the described product was filtered off, dried, then crystallized in a mixture of dioxane and ethanol or toluene and ethanol. (compound was dissolved in toluene or dioxane (10 mL) by slightly heating then filtered, and toluene evaporated to the half, cooled to room temperature and 10 mL of ethanol was added, then put it in the refrigerator to produce orange or dark orange crystals of (**6a–j**).

2.1.6d. 5-((Z)-2-(benzyloxy)-5-((E)-m-tolyldiazenyl)benzylidene)-2-(4-bromophenyl)-3-(p-tolyl)-3,5-dihydro-4H-imidazol-4-one ^{1}H NMR (δ, ppm)

2.38 (s, 3H, *p*-CH_3), 2.45 (s, 3H, *m*-CH_3), 5.21 (s, 2H, −OCH_2), 7.01–7.94 (m, 19H, Ar−H), 9.66 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):**21.36:C of p-CH_3, 21.55:C Ar-CH_3,70.79:C−OCH_2−, 112.44:C_3, 120.16:C═C, 122.70:C_1, 123.34:C_6, 124.28:C_{12}, 126.28:C_8, 126.80:C_4, 127.10:C_{22}, 127.89:$C_{14,14}{}^-$, 128.25:$C_{16,10}$, 128.37:$C_{20,20}{}^-$, 128.84:$C_{26,30}$, 129.00:$C_{15,17}$, 130.30:C_{11}, 130.90:$C_{27,29}$, 131.55:C═C_{ring}, 131.64:$C_{21,23}$, 132.07:C_{25}, 136.21:C_{13}, 138.67:C_{19}, 138.86:C_{28}, 139.00:C_9, 147.07:C_5, 152.80:C_7, 159.54:C═N, 160.34:C_2, 170.49:C═O. **Elemental Anal.** calcd for $C_{37}H_{29}BrN_4O_2$: C, 69.27; H, 4.56; N, 8.73; found: C, 69.1155; H, 4.63; N, 8.69.

2.1.6f. 5-((Z)-2-(benzyloxy)-5-((E)-m-tolyldiazenyl)benzylidene)-2-(4-bromophenyl)-3-(4-methoxyphenyl)-3,5-dihydro-4H-imidazol-4-one ^{1}H NMR (δ, ppm)

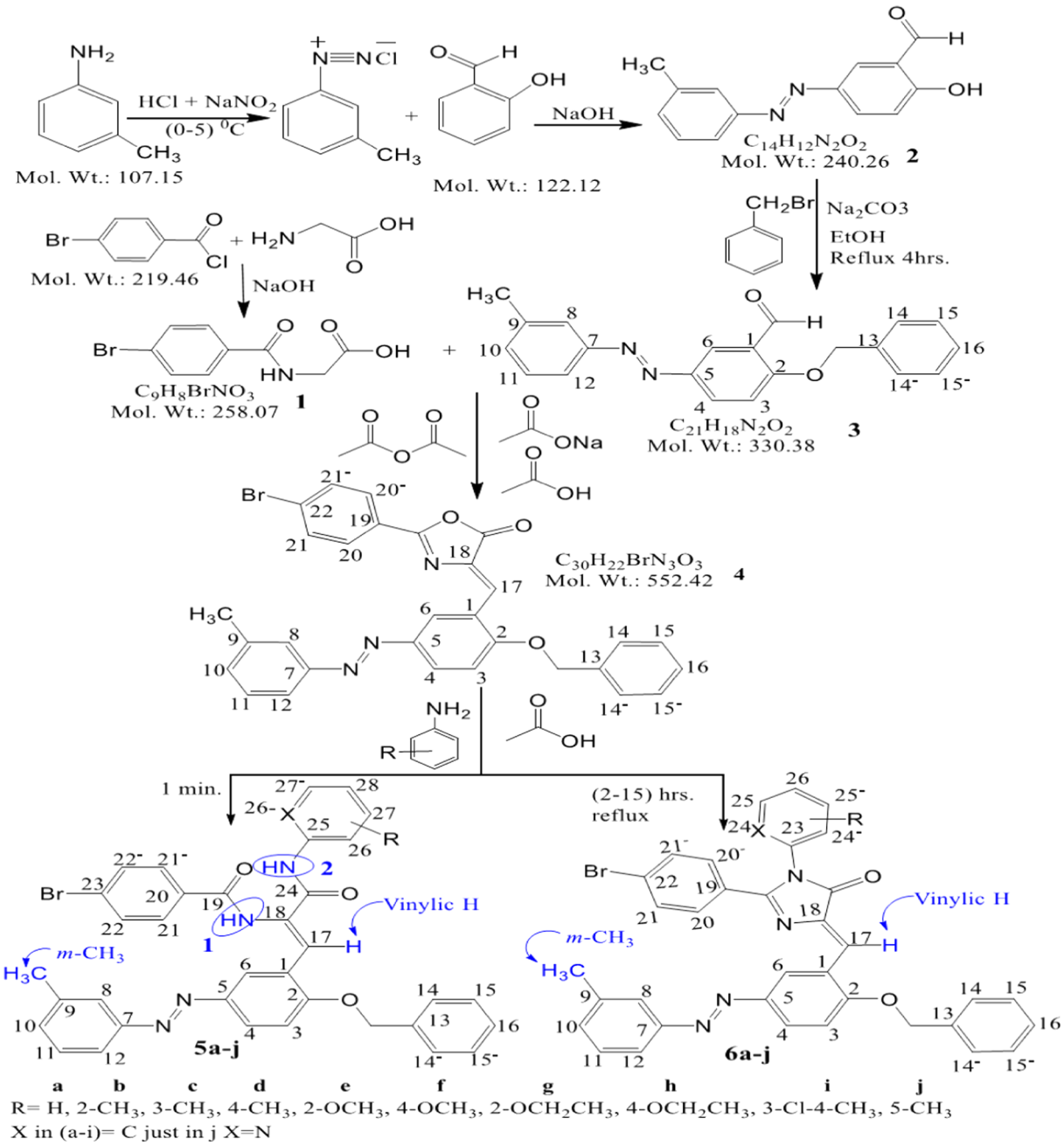
2.41–2.47 (s, 3H, *m*-CH_3), 3.83 (s, 3H, OCH_3), 5.26 (s, 2H, −OCH_2), 6.93–7.96 (m, 19H, Ar−H), 9.65 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):** 21.50:CH_3 of Ar−CH_3, 55.59:OCH_3, 70.85:C−OCH_2, 112.46:C_3, 114.94:$C_{25,25}{}^-$, 120.17:C_{17}, 122.79:C_1, 123.25:C_6, 124.32:C_{12}, 126.32:C_4, 126.77:C_8, 127.25:C_{22}, 127.34:$C_{14,14}{}^-$, 127.43:C_{16}, 127.90:C_{10}, 128.26:$C_{20,20}{}^-$, 128.33:$C_{24,24}{}^-$, 128.69:$C_{15,15}{}^-$, 128.84:C_{23}, 128.98:C_{11}, 130.88:C_{18}, 131.69:$C_{21,21}{}^-$, 136.17:C_{13}, 138.85:C_{19}, 139.01:C_9, 147.13:C_5, 152.85:C_7, 158.97:C_{26}, 159.63:C═N, 160.33:C_2, 170.72:C═O. **Elemental Anal.** Calcd. for $C_{37}H_{29}BrN_4O_3$: C, 67.58; H, 4.45; N, 8.52; found: C, 67.2314; H, 4.50; N, 8.46.

2.1.6h. 5-((Z)-2-(benzyloxy)-5-((E)-m-tolyldiazenyl)benzylidene)-2-(4-bromophenyl)-3-(4-ethoxyphenyl)-3,5-dihydro-4H-imidazol-4-one ^{1}H NMR (δ, ppm)

1.41–1.45 (t, 3H, CH_3 of −OCH_2CH_3), 2.46 (s, 3H, *m*-CH_3), 4.01–4.07 (q, 2H, OCH_2 of −OCH_2CH_3), 5.25 (s, 2H, −OCH_2), 6.91–7.95 (m, 19H, Ar−H), 9.64 (s, 1H, vinylic H). **C^{13}-NMR(δ, ppm):** 14.56:CH_3 of OCH_2CH_3, 21.61:C Ar-CH_3, 63.37:CH_2 of OCH_2CH_3, 70.84:C−OCH_2, 112.46:C_3, 115.41:$C_{25,25}{}^-$, 120.16:C_{17}, 122.73:C_1, 123.26:C_6, 124.33:C_{12}, 126.30:C_4, 126.78:C_8, 127.16:C_{22}, 127.26:$C_{14,14}{}^-$, 127.91:C_{16}, 128.26:C_{10}, 128.34:$C_{20,20}{}^-$, 128.64:$C_{24,24}{}^-$, 128.84:$C_{15,15}{}^-$, 128.98:C_{23}, 130.89:C_{11}, 131.53:C_{18}, 131.68:$C_{21,21}{}^-$, 136.18:C_{13}, 138.87:C_{19}, 139.01:C_9, 147.12:C_5, 152.84:C_7, 159.05:C_{26}, 159.64:C═N, 160.33:C_2, 170.72:C═O. **Elemental Anal.** Calcd. for $C_{38}H_{31}BrN_4O_3$: C, 67.96; H, 4.65; N, 8.34; found: C, 67.70; H, 4.31; N, 8.27.

Table 2
Some physical properties and IR data for the synthesized azo-imidazolones (**6a–j**).

Prod.	R	Chemical formula	Time h	M.P	% Yield	IR str.cm^{-1}	
						C═O	C═N
6a	H	$C_{36}H_{27}BrN_4O_2$	2	266–267	94	1718	1635
6b	2-CH_3	$C_{37}H_{29}BrN_4O_2$	2	165–166	83	1751	1680
6c	3-CH_3	$C_{37}H_{29}BrN_4O_2$	2	252–253	89	1712	1635
6d	4-CH_3	$C_{37}H_{29}BrN_4O_2$	2	314–315	94	1724	1635
6e	2-OCH_3	$C_{37}H_{29}BrN_4O_3$	2	247–248	89	1724	1670
6f	4-OCH_3	$C_{37}H_{29}BrN_4O_3$	2	218–219	92	1726	1635
6 g	2-OCH_2CH_3	$C_{38}H_{31}BrN_4O_3$	2	235–237	86	1722	1635
6 h	4-OCH_2CH_3	$C_{38}H_{31}BrN_4O_3$	2	211–212	98	1722	1627
6i	3-Cl-4-CH_3	$C_{37}H_{30}BrClN_4O_2$	10	200–201.5	92	1710	1633
6j	5-CH_3-pyridine	$C_{36}H_{28}BrN_5O_2$	15	195–196	81	1716	1674



Scheme 1. Stepwise chemical reaction for synthesis of 4, (5**a–j**) and (6**a–j**). Note; The numbers (1,2,3,…,28) indicated on the structures are used just to interpreting the ^{1}H NMR and ^{13}C NMR spectra.

2.2. Biological evaluation

2.2.1. Antimicrobial activity

2.2.1.1. Diffusion method. The antimicrobial activity was performed by disc diffusion method [38]. Some of the synthesized chemicals were tested in vitro for their antimicrobial activity against two types of bacterial strains namely *Staphylococcus aureus* and *Escherichia coli*, Known antibiotics like Azithromycin and metronidazole were used for comparison. The inhibition zones were measured in millimeters for different concentrations (200, 400, 600 and 800) (μg/mg in KBr) Fig. 1, which were prepared under pressure for each compound Table 3**.**

2.2.1.2. Dilution method for determination of (MIC). The tube macro dilution was used for measuring the minimum inhibition concentration (MIC) some of the synthesized organic compounds. The procedure was conducted by using the spectrophotometer technique at 600 nm [39]**.** The following dilutions were prepared for each compound (200, 400, 600, 800 and 1000) (μg/mL) Table 4.The results were compared with metronidazole and azithromycin as control. *S. aureus and E. coil* were used as the standard bacterial strains. The MIC was measured for organic materials which inhibited bacterial growth, against control sample which consisted of 2 mL of nutrient broth and 40 μL of activated culture of bacterial suspension as clarified in Table 4, and then it was incubated at 37 °C for 20 h. The next day optical density (OD) was recorded as shown in Table 5.

2.2.2. Creation of burn wounds

2.2.2.1. Animal care. The experiment and data collection were performed at the Department of biology, University of salahaddin, albino wistar female rats weighing 200 ± 10 g were utilized for this

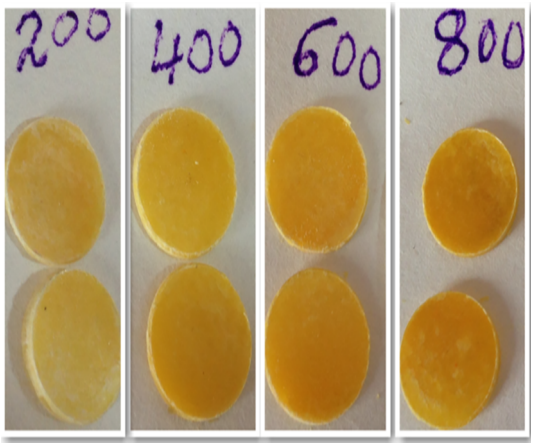


Fig. 1. Prepared known concentration of each disc (12 mm).

Table 3
preparation of difference concentration for each disc.

Conc. (µg)	Conc. Of stock (mg) 10 mg chemicals + 490 mg KBr	KBr mg	Final wt of each disc (mg)
200	10	290	300
400	20	280	300
600	30	270	300
800	40	260	300

Table 4
Preparation of test samples.

Stock solution 8000 µg/mL (0.04 g in 5 mL DMSO)				
No.	Concentration (µg/mL)	Chemical Volume (µL)	Nutrient broth volume (mL)	Bacterial suspension volume (µL)
1	200	50	1.91	40
2	400	100	1.86	40
3	600	150	1.81	40
4	800	200	1.76	40
5	1000	250	1.71	40

DMSO; dimethylsulfoxide.

experimental model in which, three rats were housed in individual cage (40 × 25 × 20) cm. The standard care and condition like photoperiod lighting conditions (12 h light/dark), temperature control (24 ± 2)°C and had free access to food and water at all times were fixed. The experimental procedure was carried out according to the ethics committee on animal experimentation (DIRECTIVE 2010/63/EU).

2.2.2.2. Burn procedure. The rats were acclimated to the laboratory for 7 days prior to beginning the study. Initially, 18 rats (n = 3) were weighed and anesthetized with a mixture of 10% ketamine and 2% xylazine (90 + 10) mg/kg body weight intramuscularly. After all rats accurately anesthetized the hair were removed by electrical clipper. Thermal injury was induced by heating 10 mm a solid stainless steel bar Fig. 2(e) on Bunsen burner until the temperature reached between (160–180)°C, which was measured with a thermometer simultaneously. Approximately 4 cm^2 areas on the dorsal proximal region of the animal skin contacted with the bar twice side by side for 5 *sec*. to produce thickness burn wounds. Fig. 2(a and b). Corresponded to the high temperature of the bar no pressure applied on the animal skin used in the burn induction [25,40].

2.2.2.3. Infection. In order to show their inhibitory roles in vivo antimicrobial activity and in wound healing assay of the synthesized compounds 4, 5d and 6d, after 24 h a burn occurred, the burnt rats were infected by activated culture of *S. aureus* [26] which were stored for 18 h. In Müller-Hinton agar medium and then the turbidity of bacterial suspensions were adjusted in sterile distilled water to match 0.5 McFarland standards (10^8 CFU/mL) approximately, then the activated culture swabbed over the wound of each injured rat Fig. 2(c) and left to growth up for 24 h.

2.2.2.4. Cream preparation. Three creams of the compounds 4,5d and 6d in a concentration of (1% w/w) were prepared by dissolving 0.05 g of each compound in 2 mL acetone in crucible and adding then 5gm of pure paraffin wax added, heated on the sand bath with gentle stirring to liquidity the wax and also to remove the acetone from the mixture, then cooled to room temperature with stirring to produce homogenous cream, they were sterilized in autoclave under steam heat at 121 °C (15 Ib/in^2) typically for 15 min, then cooled with stirring Fig. 3.

2.2.2.5. Wound healing. Burnt rats were randomly divided for four main groups test (4,5d and 6d), positive control (pure paraffin wax), negative control (no treatment) and stander group (Ucederm 1%) (n = 3), the treatment started after 24 h of infection, the injured area in each wounded rat swabbed by these creams in a single dose during 24 h the percentage of wound closure was monitored and calculated for each group and the result shown in Figs. 4 and 11.

2.2.3. Oral acute toxicity/single dose toxicity study

In order to examine the toxicity ability of each (4, 5d and 6d) in living system. Wistar albino female rats (weighing between 210 and 230 g) were selected for acute oral toxicity test by up and down procedure (OECD Test Guidelines 425). The animals were acclimatized for one week and fasted overnight prior to dosing.

2.2.3.1. Dose preparation. The required amount of the tested compounds (mg/kg) body weight, dissolved in acetone and then the vehicle (corn oil) added, heated gently with stirring to homogenize the mixture and removing acetone, Table 6.

2.2.3.2. Dose administration. According to (OECD/OCDE 425–27), three animals were weighed, the limited test (2000 mg/kg) body weight is administered in a single dose or twice dose in 24 h, by gavage using a stomach tube for each compound, and corn oil was used as vehicle and control Table 6. After dose administration, the animals were individually monitored for first 6 h (each 30 min), periodically during the first 24 h and daily for a total of 14 days. Further observation such as behavioral changes and Changes in eyes, skin and fur were recorded for each rat in comparison to the control during the experiment. On day 15, the rats were intramuscularly anesthetized with a mixture of ketamine10% and xylazine2%, sacrificed, and were examined macroscopically for their external surface, abdominal organs and thoracic organs.

2.2.4. FRAP (Ferric reducing antioxidant Power) assay

In order to measure the ferric reducing activity of our samples, assay was conducted according to [41]. The assay, originally proposed by [42]. The FRAP solution was freshly prepared by combination of acetate buffer (0.3Mol/L) (pH = 3.6), 2,4,6-tripyridyltriazine (TPTZ) (0.01Mol/L in HCl (0.04Mol/L) and $FeCl_3$ (0.02Mol/L) (10:1:1) by volume, respectively. The assay was carried out by placing 100 µL of the tested chemicals (Conc. 1 mg/mL) and 3 mL of FRAP reagent in a test tube, the samples were continuously shaked for 15 min. Then the absorbance was recorded at 592 nm. Table 7. The standard curve of ascorbic acid was prepared using different concentrations for comparison Fig. 5. The FRAP value for each compounds calculated by the following equation.

Table 5
Antimicrobial activity of the synthesized compounds against Staphylococcus aureus and Escherichia coil.

Compounds		Concentration of chemicals (μg/mL) (conc. vs abs.)				
4	*S. aureus*	200	400	600	800	1000
	E. coli	1.631	0.610	NG	NG	NG
5a	*S. aureus*	1.758	1.300	0.836	0.882	0.795
	E. coli	1.340	0.876	0.398	0.158	NG
5c	*S. aureus*	1.174	0.650	NG	NG	NG
	E. coli	1.674	1.058	0.688	0.273	NG
5d	*S. aureus*	1.424	0.762	0.375	NG	NG
	E. coli	0.935	NG	NG	NG	NG
5 h	*S. aureus*	0.747	0.075	0.123	NG	NG
	E. coli	1.128	0.983	0.631	0.358	0.269
5i	*S. aureus*	1.525	0.775	0.487	0.286	NG
	E. coli	1.038	0.695	0.205	NG	NG
6a	*S. aureus*	1.481	0.985	0.643	0.500	NG
	E. coli	1.070	0.897	0.376	NG	NG
6c	*S. aureus*	1.725	1.075	0.817	0.404	0.005
	E. coli	1.256	0.745	0.234	NG	NG
6d	*S. aureus*	1.341	0.858	NG	NG	NG
	E. coli	1.524	0.762	NG	NG	NG
6h	*S. aureus*	1.114	0.226	NG	NG	NG
	E. coli	1.574	0.450	NG	NG	NG
Azithromycin	*S. aureus*	1.375	0.040	NG	NG	NG
	E. coli	1.203	NG	NG	NG	NG
Metronidazole	*S. aureus*	1.400	0.11	NG	NG	NG
	E. coli	1.384	0.028	NG	NG	NG

NG; No growth of bacterial strain.

$$FRAP\ \ value\ \ of\ \ sample\ \ (\mu M) = Abs.\ (sample) * \frac{FRAP\ \ value\ \ of\ \ stander\ \ (\mu M)}{Abs.\ \ of\ \ stander}$$

3. Data analyses

Data analysis was performed using Microsoft Excel 2010, Graphpad prism6, UVprobe 2.43 and IRsolution software.

4. Result and discussion

4.1. Chemistry

The improvement of advanced materials from simple molecules requires their modification to develop functional properties. Which was desirable for some bio-related uses, such as drug, biomedical, food and electronical applications.

Herein, we have synthesized hybrid molecules involving azo linkage and heterocyclic moiety (oxazolones or imidazolones) Scheme 1, Oxazolone **4** was synthesized in buffered solution via modified Erlenmeyer–Plöchl azlactone synthesis as the 4-bromobenzoylglycine **1** is interfered to an azo-benzylated benzaldehyde **3**. This modification greatly enhanced the yield and reducing time from hours to minutes. The resulting product was unbalanced molecule Scheme 2 due to the azo moiety and benzyloxy group were in the same side of azlactone ring, respectively, which serving us to synthesis a series of azo-benzamide **(5a–j)** in accordance to green synthesis protocol. The reactive azo-oxazolone refluxed in acetic acid 99.9% in the presence of various primary aromatic amines involves fast ring opening (within seconds) even faster than under microwaves [36,43]**.** Finally, azo-benzamide derivatives were refluxed in glacial acetic acid resulting azo-imidazolone derivatives **(6a–j)** by intramolecular cyclodehydration. Notably, the presence of an electron-withdrawing part in the aniline significantly decreases the rate of reaction Table 2.

The structural confirmation of some synthesized compounds were

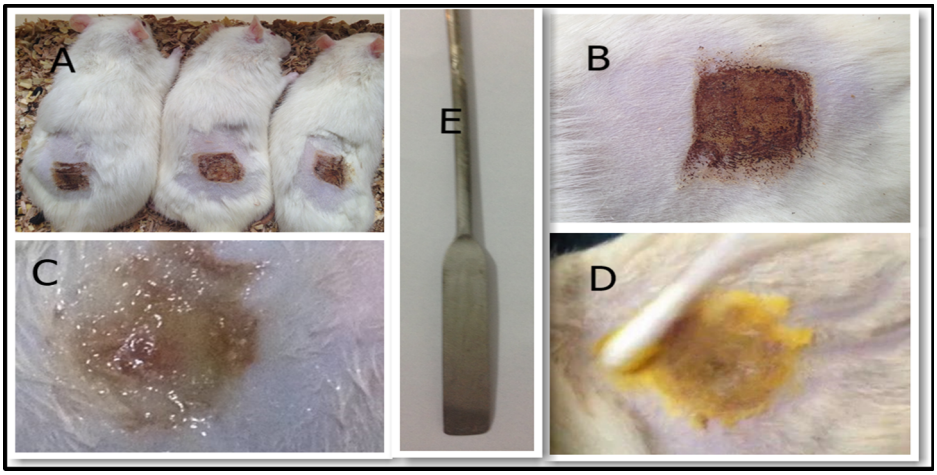


Fig. 2. Photographs of the appearance of different wounds (A and B) are on burnt day, (C) infected burn with *S. aureus*, (D) treatment of burnt wound, (E) 10 mm bar used for making burn.

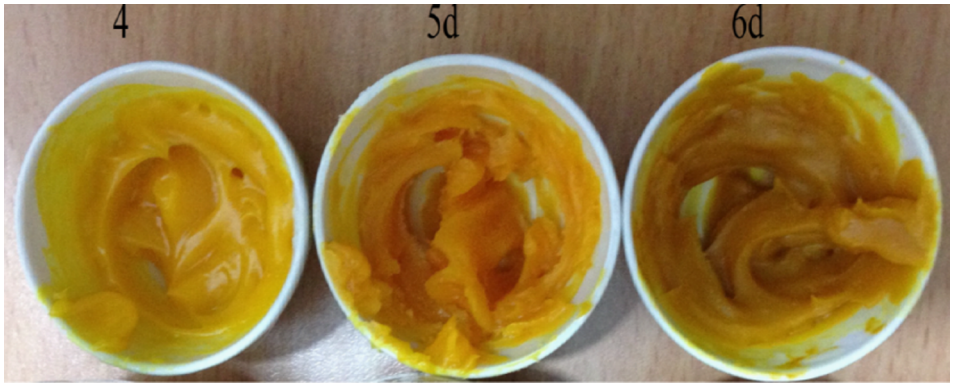


Fig. 3. Prepared creams.

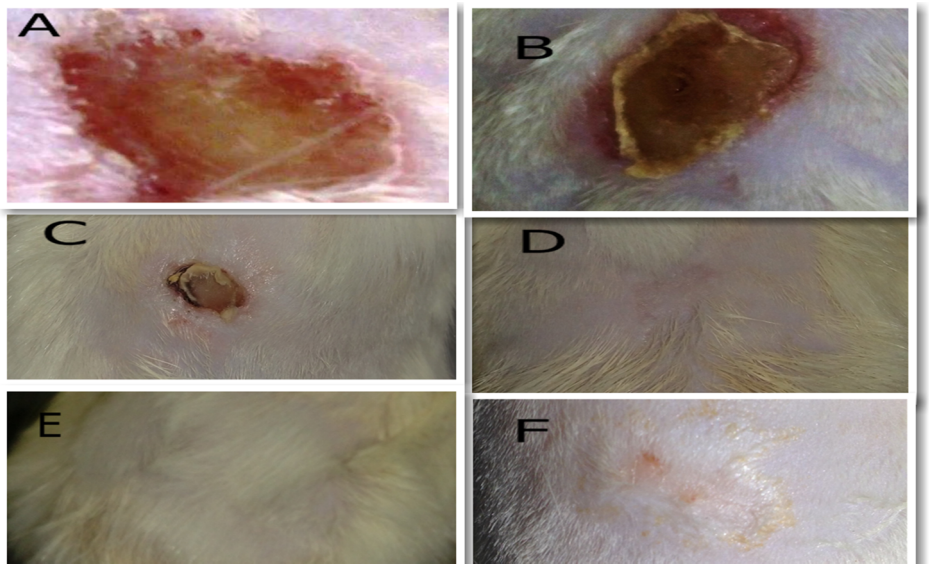


Fig. 4. Differences in healing progression of wounds at days 0 (A), 4 (B), 10 (C), 15 (D) and 25 (E). Note F is representing shaved E.

Table 6
Dose preparation and dosing per rat.

Conc. mg/kg	Acetone (mL)	Vehicle (mL)	No. of doses in 24 h	No. of used rat	lethality
2000	2	3	1	1	No
2500	2	3	1	1	No
3000	2	3	1	1	No
3500	4	5	2	1	No
4000	4	5	2	1	No
4500	4	5	2	1	No
5000	4	5	2	3	No

Table 7
Anti-oxidant activity some of the synthesized compounds.

Comp.	Abs.	FERAP value		
		µM	µM/mL	µM/gm
4	0.213	7.66	76.6	766
6d	0.081	2.91	29.1	291
6f	0.138	4.96	49.6	496
6 h	0.171	6.15	61.5	615
5d	0.037	1.33	13.3	133
5f	0.106	3.81	38.1	381
5 h	0.216	7.77	77.7	777
Ascorbic acid	2.327	83.705	837.05	8370.5

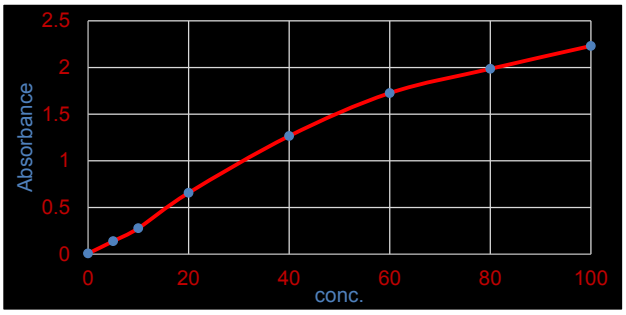
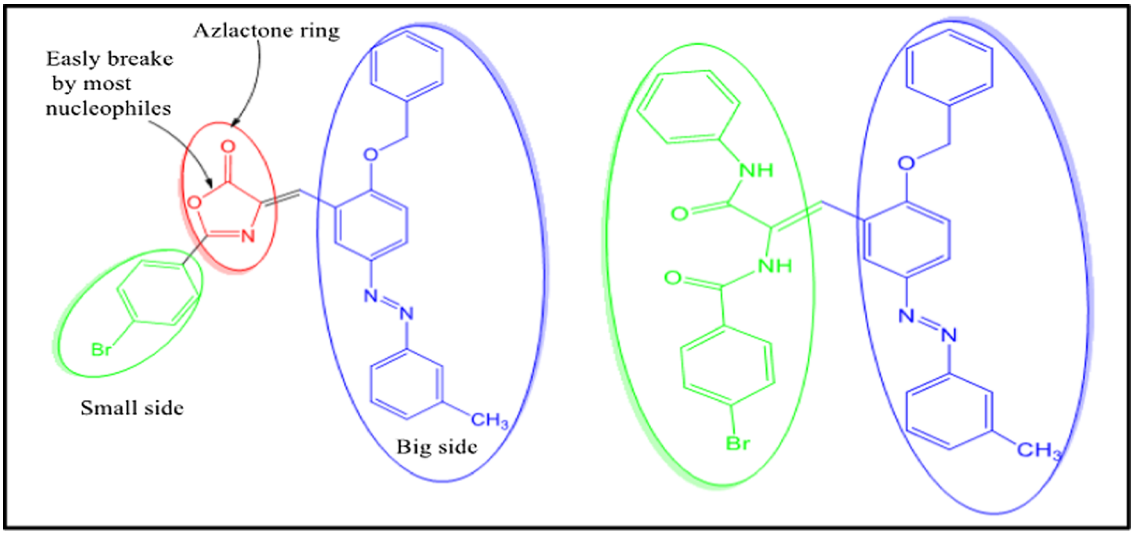


Fig. 5. The standard curve of ascorbic acid.

made by their physical properties and spectral data analysis such as (FT-IR, ^{1}H NMR, C^{13}-NMR and CHN analysis) see Figs. 14–22. The structure of the synthesized compounds (4, 5d, 5f, 5 h, 6d, 6f and 6 h) adequately identical with the assumed structures. IR spectra was used to monitor the carbonyl shifting from compound **(2)** to compound **(6)** Fig. 6.

The absorption spectra of the benzamide azo dyes **(5a–j)** Fig. 7 and imidazolone azo dyes (**6a–i)** Fig. 8 were recorded in dioxane at room temperature in quartz cuvette (C = 50 ppm). Generally, two absorption bands at 400–410 and 290–300 nm available for azo compounds corresponding to n–π* and π–π* transitions, respectively [44]. The UV–visible spectra azo-benzamide derivatives significantly differ from those



Scheme 2. Unbalanced azo-oxazolone 4 and approximately balanced benzamide 5a.

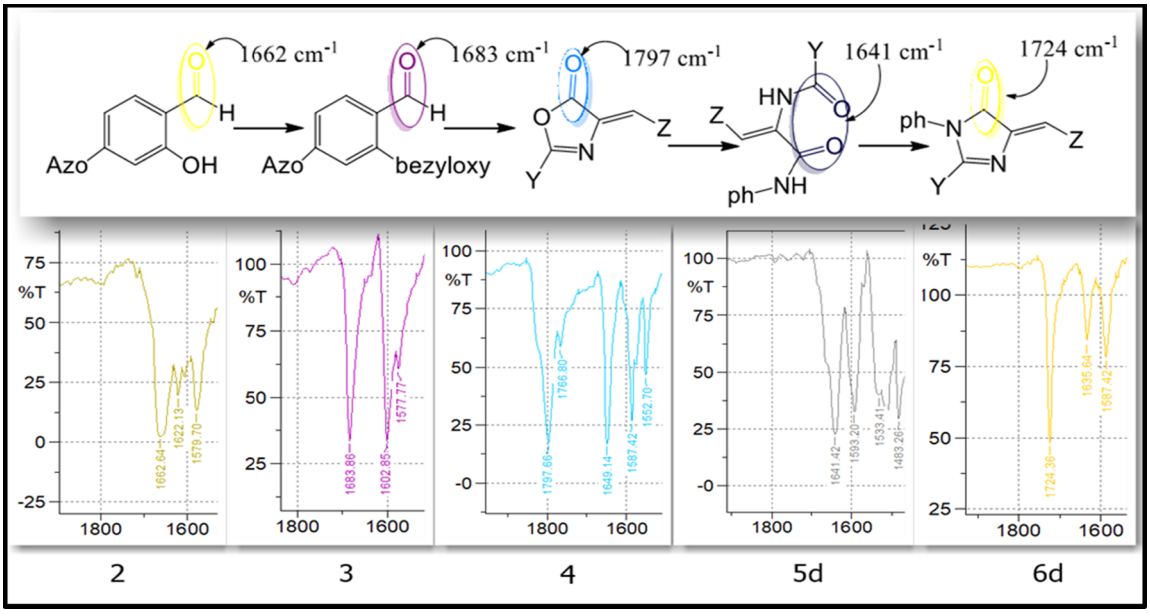


Fig. 6. FT-IR charts of C═O *str*. shifting from compound 2 to compound 6 series.

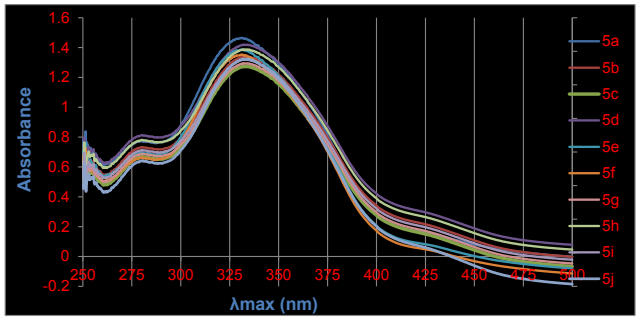


Fig. 7. UV–Visible spectra of azo-benzamidederivaties (**5a–j**).

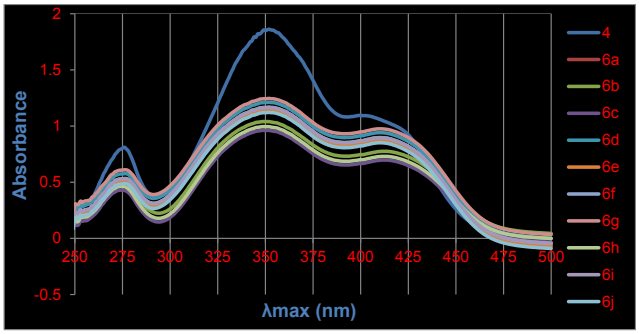


Fig. 8. UV–Visible spectra of azo-imidazolone derivatives (**6a–j**) and compound 4.

showed for azo-imidazolone derivatives, the azo-imidazolone dyes show three intense bands in the (270–280, 340–360 and 410–420) nm regions. The (410–420) nm band of the parent imidazolone ring disappeared from the determinate region for the azo-benzamide dyes [22]. It was noted from both figures that the approximately same spectrum observed for each compounds and the absence of red shift due to the all substitutes far away from the azo group, which means there is no electronic effect on the azo group. Finally, it should be emphasized that all compounds with long π-conjugated bonds present absorption in the

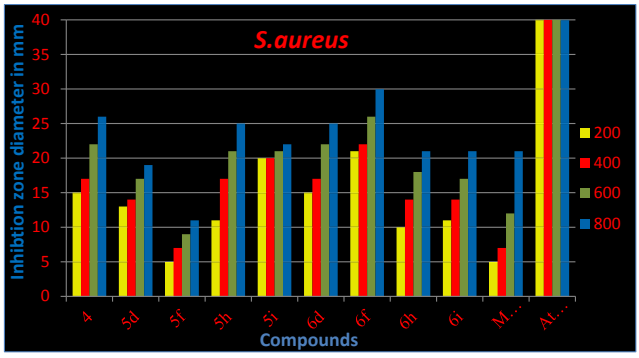


Fig. 9. In vitro antimicrobial activity of screened compounds against *S. aureas.*

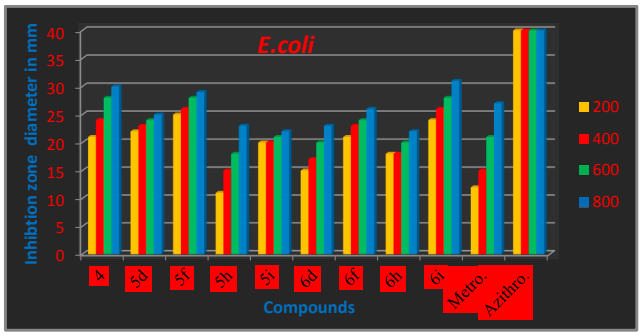


Fig. 10. In vitro antimicrobial activity of screened compounds against *E. coli.*

Table 8
Clarification of used mg for each treated group.

Treatment groups	Total wt of cream 1% (g)	Remained after treatment (g)	Used cream for treatment	day used for healing	cream of dose/ day	mg of each comp. used/ day
4	5	2.02	2.98	13	0.23	2.3
5d	5	1.92	3.08	16	0.19	1.9
6d	5	1.3	3.7	14	0.26	2.6
+	15	4.52	10.48	25	0.42	4.2
uced.	15	7.3	7.7	22	0.35	3.5

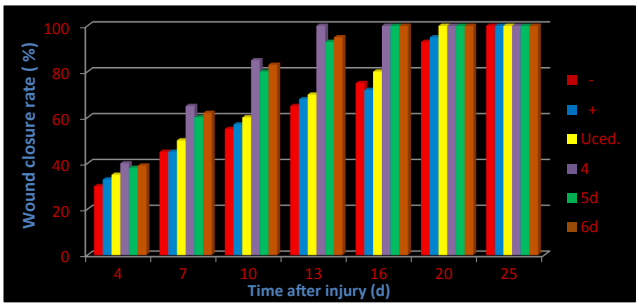


Fig. 12. The wound closure rate of different wounds on different days; −: represent infected wounds without treatment, +: represent infected wounds treated with pure paraffin wax, uced.: represent infected wounds treated with Ucederm drug, (4, 5d and 6d) groups: represent infected wounds treated with synthesized compound 4, 5d and 6d respectively.

visible region which may serve as an efficient photo switch in biological media that require low-energy light, because, in case more energetic light could destroy the system [22].

4.2. Biological activity

4.2.1. Antimicrobial activity

The synthesized chemicals (4, 5d, 5f, 5 h, 5i, 6d, 6f, 6 h and 6i) were screened against *S. aureus as* a Gram positive and *E. coli* as a Gram negative bacterial strains by diffusion and dilution methods. The results of antibacterial screening as in Figs. 9 and 10 reveal that all tested compounds displayed better activity toward *E. coli* than *S. aureus*, In our experiments, the tested compounds show relatively stronger antimicrobial activity in dilution method than diffusion method. This difference due to the large surface area available for contacting between chemicals and bacterial strains, ultimately kill or inhibit growth maximum number of bacterial strains. In both methods the antimicrobial intensity was concentration dependent. The maximum zone inhibition activity of the tested compounds was showen at the concentration

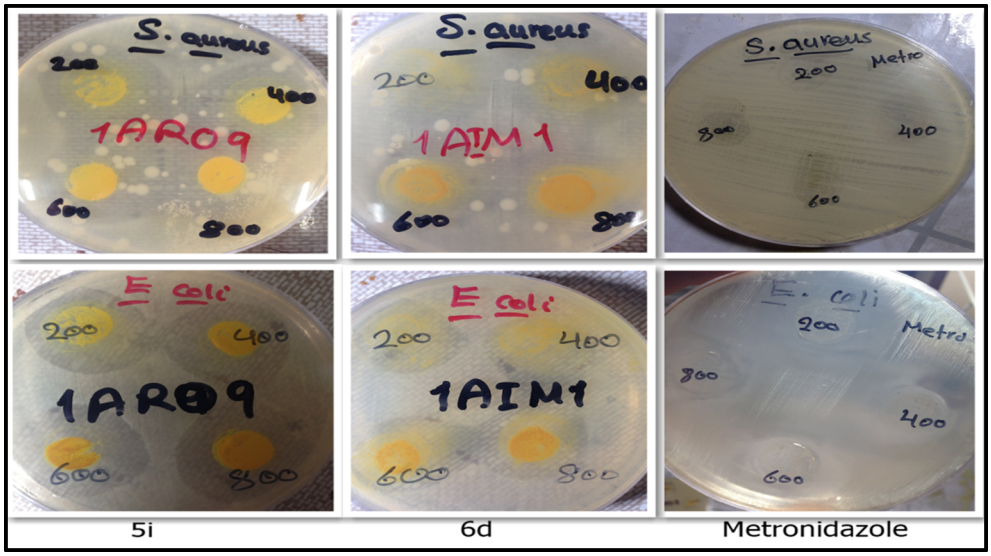


Fig. 11. Antimicrobial activities of 5i, 6d and metronidazole by disc diffusion method with difference concentrations (200, 400, 600 and 800) µgm.

Table 9
Acute toxicity hazard categories and classification System for (approximate) LD50/LC50 values defining the respective categories (OECD 1998b; updated OECD, 2001).

Acute Toxicity Route	Toxicity Class 1	Toxicity Class 2	Toxicity Class 3	Toxicity Class 4	Toxicity Class 5
Oral					
LD50 Values (mg/kg) [approximate]	≤5	> 5 ≤ 50	> 50 ≤ 300	> 300 ≤ 2000	> 2000 ≤ 5000

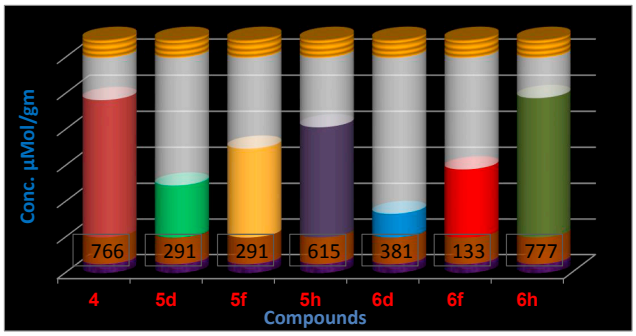


Fig. 13. Antioxidant activity of tested compounds measured by FRAP assay.

(800 µg) between (20–30) mm against *S. aureus* Fig. 9 and (22–31) mm against *E. coil* Fig. 10. Compounds 4, 5 h, 5i, 6d, 6f and 6i showed highest activity (22–31) mm) against both microorganisms. The rest of the studied compounds exhibited good to moderate activity (11–25 mm) against these microorganisms.

4.2.2. Wound healing assays

Encouraged by the in vitro results of the screened above, the skin wounds infected with *S. aureus* on animal model was performed to evaluate whether these chemicals had the competence to repair the wounds with Gram-positive bacterial infections in vivo and on the other hand, in the last decades one of the most common pathogens isolated from burn patients *was S. aureus.* The strong pathogenicity of *S. aureus is* due to its ability to produce various exotoxins which cause to non-healing residual wounds and skin grafting failure [45]**.** The active compounds 4, 5d and 6d were subjected to further study in the form of cream (1%) in comparison to ucederm (1%) as a stander drug. First, approximately 4 cm^2 wound dressing were prepared, infected with *S. aureus* and treatment with different creams were done in three successive 24 h. Rats were daily treated in a single dose by approximately 2 mg used in 24 h for treated groups Table 8**.** The percentage of wound closure was monitored for each group and the results were shown in Fig. 12**.**

4.2.3. Acute oral toxicity

The 4, 5d and 6d compounds were tested first for acute toxicity on rats at concentrations (2000–5000) mg/kg body weight to determine their 50% lethal Concentration, during the experiment no lethality was recorded and the rats did not show any significant evident concerning the toxicological signs, It is clear from Tables 6 and 9 that the tested compounds were classified as potentially nontoxic [46]. At the end of the experiment, the used rats were sacrificed, the abdominal organs and thoracic organs were weighed, the changes were not considered to be associated with the toxicological changes between the control group and treated group.

4.2.4. FRAP reducing power

The antioxidant activity of seven synthesized compounds were measured by FRAP is presented in Fig. 13**.** It shows that electron donating groups enhanced reducing power. Compounds (5 h and 6 h) with OCH_2CH_3 group indicate that they is most effective electron donor and can reduce the oxidized intermediates highly reactive molecules like free radicals and reactive oxygen species of peroxidation processes. In this study, the yellow color of the test solution reduce ferric complex with TPTZ (color less) to Ferrous complex with TPTZ (violet color) changes to various shades of violet depending on the reducing power of each compound, In this assay. The higher reductive potential was determined by higher absorbance of the reaction mixture.

5. Conclusion

In the present study we have showen the combination of two excellent molecular moieties azo group and heterocyclic chromophores. The highly versatile azo-oxazolone intermediate has been synthesized in buffer solution. It is noteworthy that in comparison to classical method buffer solution afforded some advantages such as excellent yield, short reaction time and easy workup. Also, it should be noted that the azo side chain in oxazolone made extremely reactive molecule which enhance the ring opening reaction for synthesis of a series of azo-benzamides and a series of azo-imidazolone analogs, which were confirmed by proper apparatuses such as FT-IR, ^{1}H NMR, C^{13}-NMR and CHN analysis. The expected structures were in accordance with collected spectral data. The comparable in vivo and in vitro antimicrobial evaluated. All the screened compounds 4, 5 h, 5i, 6d and 6f showed good activity against *S. aureus* whereas all screened compounds were found active toward *E. coli*. On the other hand, the growth inhibition of *S. aureus* and wounds repair was achieved as results of treatment by **4, 5d and 6d** on the rats infected wound. Consequently, showed balanced between biological safety and antimicrobial activity. The results obtained for the three used compounds in comparison to the stander drug ucedem will be highly beneficial toward new powerful compounds as novel antimicrobial and repair of the wounds. Compounds 4, 5 h and 6 h showed remarkable reducing power which is significant indicator of its potential antioxidant activity. According to acute toxicity hazard categories (OECD) the tested compounds (4, 5d and 6d) are totally safe against living system.

Conflict of interest

Authors have no conflict of interest.

Acknowledgement

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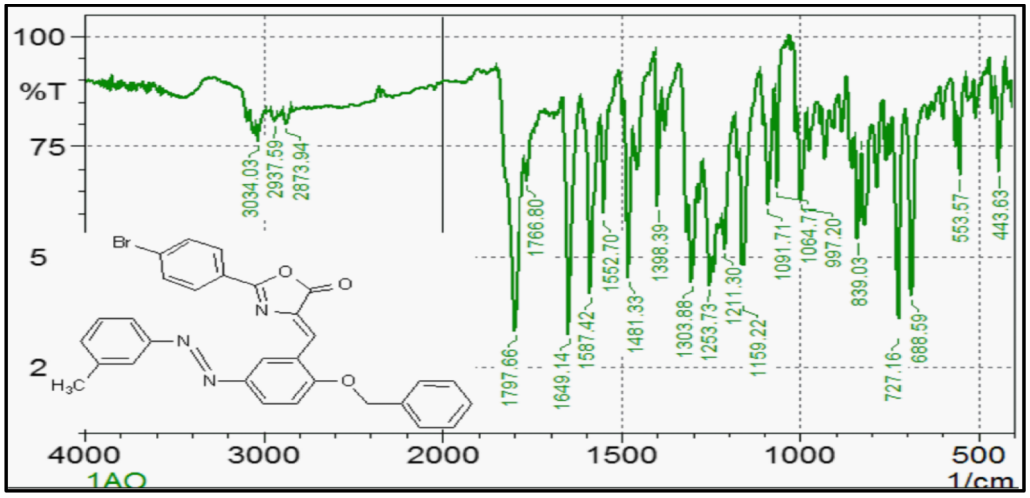

Fig. 14. Represent IR Spectrum of compound 4.

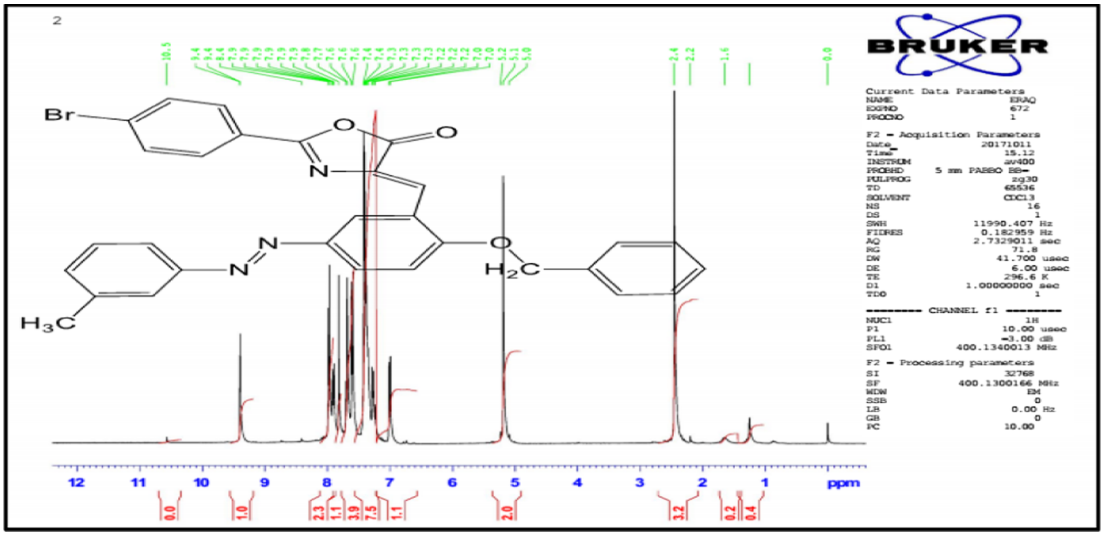

Fig. 15. Represent ^{1}H NMR Spectrum of compound (4).

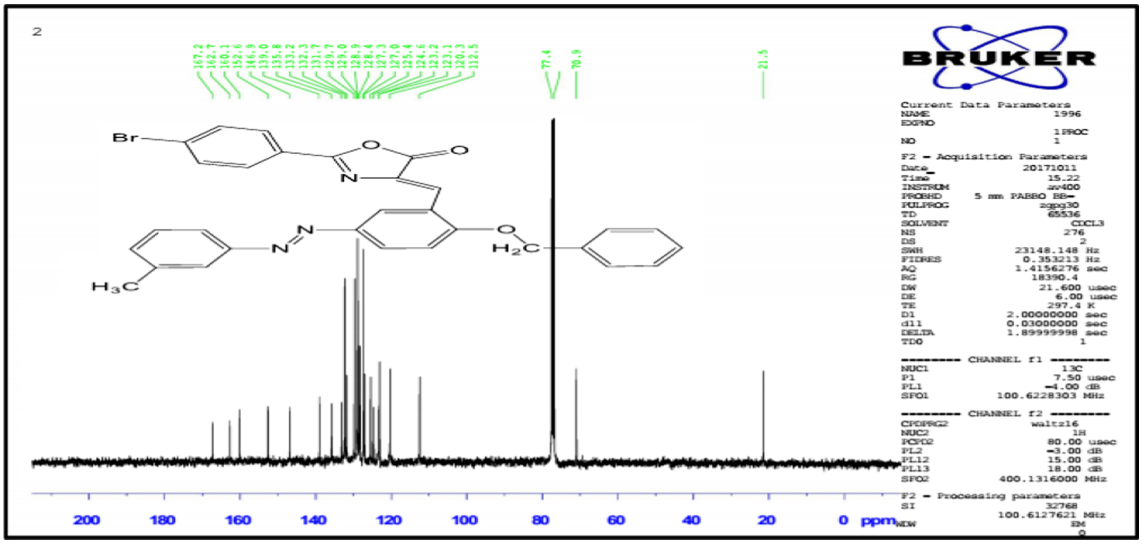

Fig. 16. Represent C^{13}-NMR Spectrum of compound 4.

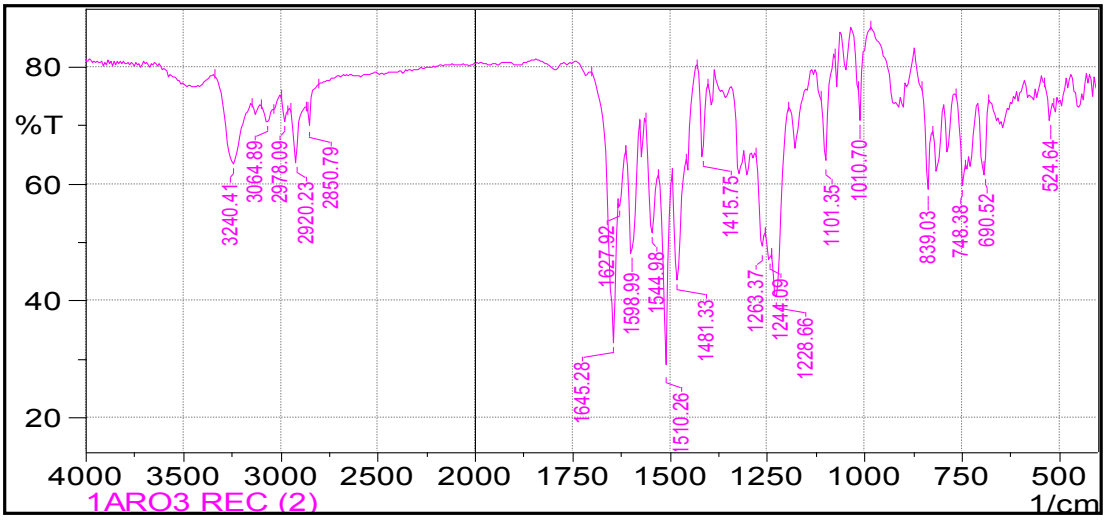

Fig. 17. Represent IR Spectrum of compound 5 h.

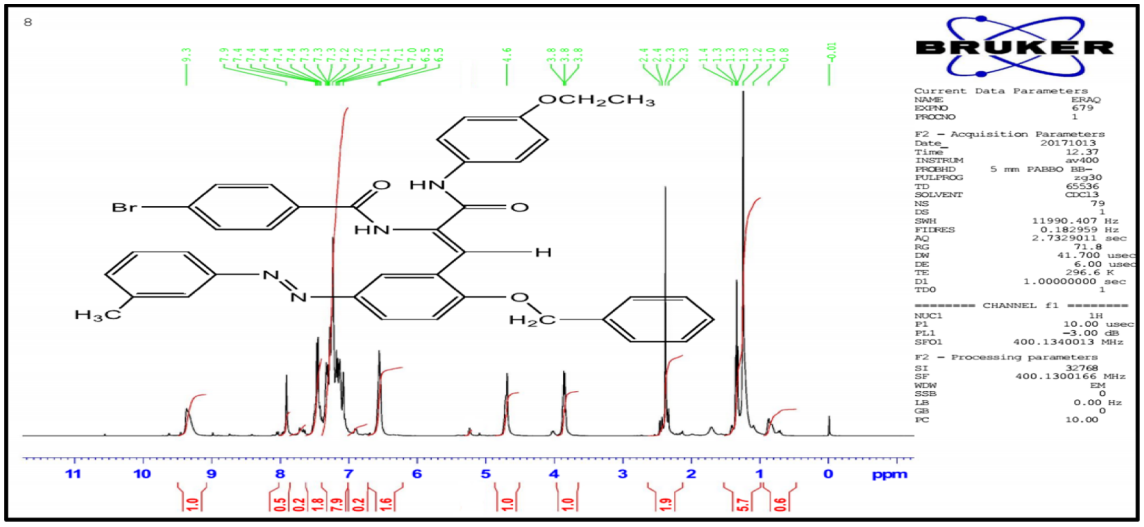

Fig. 18. Represent ^{1}H NMR Spectrum of compound 5 h.

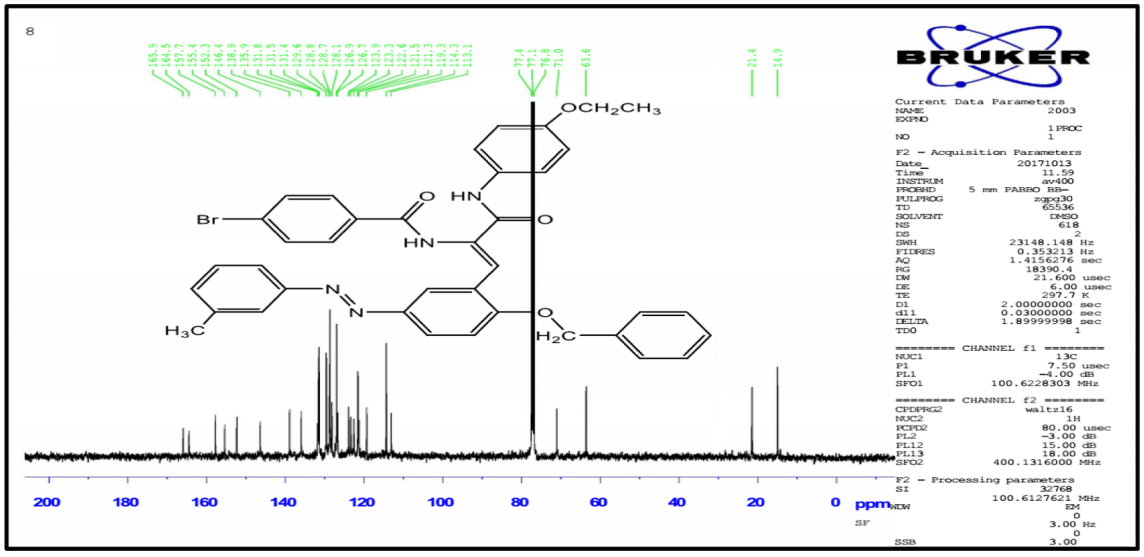

Fig. 19. Represent C^{13}-NMR Spectrum of compound 5 h.

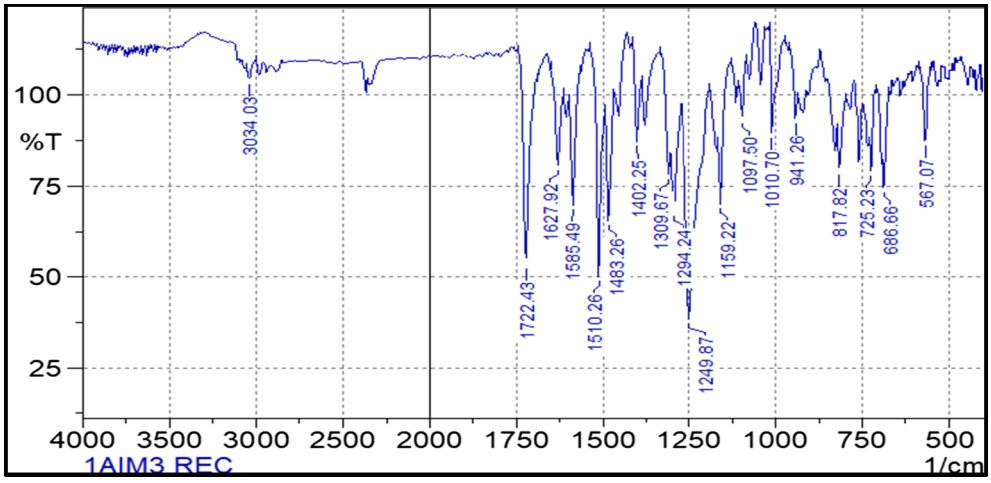

Fig. 20. Represent IR Spectrum of compound 6 g.

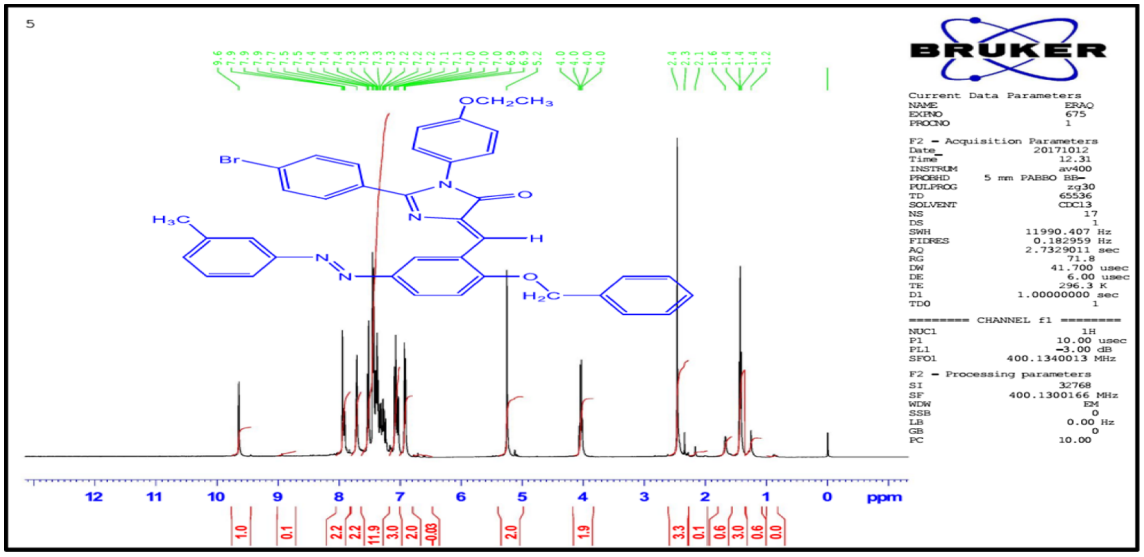

Fig. 21. Represent ^{1}H NMR Spectrum of compound 6 g.

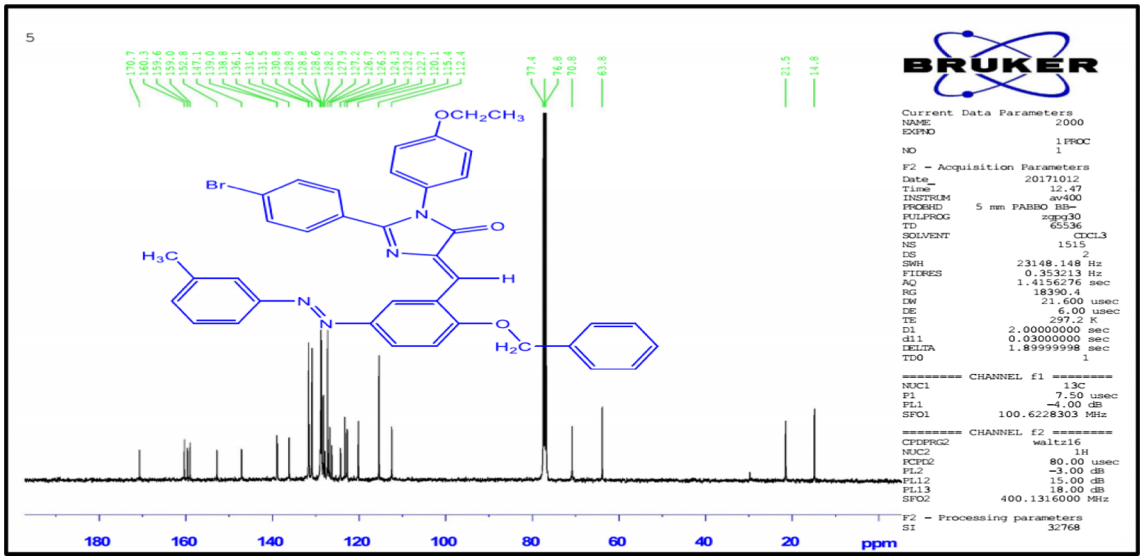

Fig. 22. Represent C^{13}-NMR Spectrum of compound 6 g.

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