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Solvent Free Synthesis of (*E*)-4-Benzylidene-3-Methylisoxazol-5(4*H*)-Ones and their Cytotoxic Screening against MCF7 Cell Line

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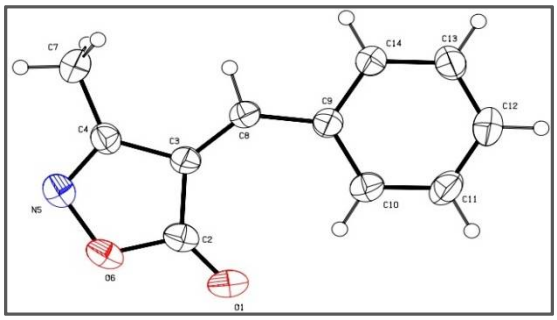
ABSTRACT

*A novel, green and efficient solvent free methods such as autoclave and microwave assisted reaction has been designed for the synthesis of (E)-4-benzylidene-3-methylisoxazol-5(4H)-one derivatives. This path emerged with many advantages such as curtailed reaction time, easy isolation of products, increased yields and eco-friendly reaction conditions. Presently, seven isoxazole derivatives were tailored by blending various benzaldehyde derivatives with ethyl acetoacetate and the new molecules were characterized by spectroscopic analysis such as IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. The newly synthesized compounds were screened for their breast cancer activity against MCF7 cell line. The compounds **4(a-g)** have shown moderate activity.*

Graphical Abstract



ORTEP diagram of the molecule **4a** with 50% probability displacement ellipsoids.

Keywords: Autoclave/Microwave reaction, aromatic aldehyde, ethyl acetoacetate, $NH_2OH.HCl$, anhydrous$ZnCl_2$.

INTRODUCTION

The most common cancer among woman is breast cancer [**1**]. It represents approximately25% of all cancers with an estimated 1.7 million new cancer cases diagnosed in 2012. When we compare breast cancer in India, China and the US, it gives an idea of the trends it is following. According to this comparison done by the *Globocan Project*;In US, one lady is dying out of six women diagnosed with breast cancer, in China, one lady is dying out of four women newly diagnosed and in India, for every 2 women newly diagnosed, one lady is dying of it [**2**].

Whenever we deal with heterocycles, isoxazole and its derivatives attracts researchers due to its unrivalled efficacy. Isoxazole is an important nitrogen and oxygen containing hetero nucleus, which display unique properties required to become an attractive moiety mainly in the agricultural field such as, larvicidal [**3**], insecticidal [**4-6**], herbicidal [**7**] and fungicidal [**8**] activities. They also, display various biological applications like antiviral [**9**], anticancer [**10-14**], antiplatelet [**15-16**], antituberculosis [**17**], analgesic [**18**], anti-HIV [**19**], antinociceptive [20], anticonvulsant and immune modulating activities [**21-23**]. Due to their excellent biological portrait, A and D-ring coalesce heterosteroids with isoxazole moieties were prepared and many of which are highly potent molecules towards antimicrobial [**24**], hypocholesterolemic and diuretic activity [**25-29**].

A number of synthetic strategies have been developed for the preparation of isoxazole derivatives such as; 1,3-dipolar cycloaddition reaction of unsaturated hydrocarbons or its derivatives with nitrile oxide which is produced by aldoxime or nitroalkanes [**30-37**] by condensing hydroxylamine with specific 1,3-dicarbonyl compounds or its 1,3-electrophilic variants [**38**], addition reaction [**39**], cyclo-condensation reaction [**40**], ring opening endo-recyclization reaction [**41**] in the presence of solvents.

Apart from this, some novel isoxazole derivatives were prepared in aqueous media and using various catalyst followed by microwave irradiation [**42**], organocatalytic bases in aqueous medium [43-48], by visible light in aqueous ethanol [**49**], by tartaric acid catalyzed in aqueous medium [50] and by using various catalysts [**51, 52**] has been reported. The methods used above are suffering from long reaction time, exhaustive isolation, poor yield, use of catalyst etc. To overcome the above problems we have developed a novel green and environmentally benign method for the synthesis of (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-one derivatives and the newly synthesized molecules were screened for their cytotoxic activity against MCF7 cell line.

MATERIALS AND METHODS

General procedure for autoclave Synthesis of (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-one (4a): Benzaldehyde **1** (0.106 g, 1 mmol, 1 equiv), ethyl acetoacetate **2** (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride **3** (0.069 g, 1 mmol, 1 equiv) and zinc chloride (0.2 equiv) were taken in an autoclave reaction container (Teflon Liner). The cloche was fixed and was stirred well for 5 minutes. Then the container inserted into the autoclave, plated over it and autoclave closed and tightened. Then it was kept inside the oven and heated at 160^{o} - 180^{o}C for 2 h. The Resultant crude product was dissolved in ethanol, and tested for completeness by monitoring TLC, which shows a single spot different from precursors spot. Finally obtained solid mass was treated with ethanol (20 mL), and evaporated for dryness which yields the title compound with 95% yield. Mp: 139-142 °C (lit 141-143°C) [51]. The same procedure was followed for all the derivatives.

General procedure for microwave assisted synthesis of (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-one (4a): Benzaldehyde (0.106 g, 1 mmol, 1 equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (0.1 equiv) were taken in a 50ml RB flask and mixed thoroughly to get homogeneous solution. Then the flask was placed inside the microwave oven and the reactants irradiated at 2.45 GHz over the period of 2 minutes. Then the flask was allowed to cool and tested for completion of reaction. Since the reaction

does not progressed, the flask was again irradiated for another 2 minutes and repeated the same over a period of 18-20 min. The Resultant crude product was dissolved in ethanol, and tested for completeness by monitoring TLC, which shows a single spot different from precursors spot. Further the crude product purified by column chromatography using ethyl acetate: n-hexane (2:8) as an eluent which yields 79% and recrystallized by hot ethanol which is used for melting point detection.

Further, obtained products were identified by IR, ^{1}H NMR, ^{13}C NMR and Mass spectral studies. For the comparison yields in autoclave and microwave reaction see table 1. As mentioned in the table 1, the product yield obtained by autoclave reaction is more compare to microwave assisted synthesis. Initially we did 1:1, 1:2 and 1:3 ratios of benzaldehyde and ethyl acetoacetate to optimize the reaction. With respect to different proportion, autoclave reaction with 1:3 proportion yields paramount yield compare to microwave synthesis.

(*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-one (4a): Light yellow solid, M.p:139-142 °C, obtained from Benzaldehyde (0.106 g, 1 mmol, 1 equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum (KBr), ν: 1757, 1638, 1112, 1213, 875, 768 cm^{-1}. ^{1}H NMR spectrum (400 MHz, $CDCl_3$), δ, ppm (*J*, Hz): 2.41 (3H, s, CH_3), 7.40 (1H, s, PhC<u>H</u>CCNOC), 7.50-7.56 (3H, m, H Ar), 7.89-7.95 (2H, d, H Ar, *J* = 8.8). ^{13}C NMR spectrum (400 MHz, $CDCl_3$), *δ*, ppm: 17.1, 125.9, 127.4, 127.9, 127.8, 133.3, 152.2, 165.1, 176.0. MS, *m/z*: 187.04 (M^+), 147.01, 128.06, 89.02, 81.02.Found, %: C 70.59; H 4.88; N 7.46. $C_{11}H_9NO_2$. Calculated, %: C 70.58; H 4.85; N 7.48.

(*E*)-4-(4-chlorobenzylidene)-3-methylisoxazol-5(4*H*)-one (4b): Orange yellow solid, M.p:127-131°C, obtained from 4-chlorbenzaldehyde (0.140g, 1 mmol, 1equiv) ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum, (KBr) ν: 1742, 1546, 1227, 1128, 831, 765 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.4 (s, 3H, CH_3), 7.40 (s, 1H, vinyl), 7.57-7.59 (d, 2H, ArH, *J* = 8.0), 7.89-7.91 (d, 2H, ArH, *J* = 8.0); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.3, 126.2, 128.8, 129.6, 133.7, 135.7, 151, 165.1, 176.2. MS, m/z: 221.01 (M^+), 223.02, 222.02, 224.02, 223.03. Found, %: C 59.59; H 3.67; N 6.31. $C_{11}H_8O_2NCl$. Calculated, %: C 59.61; H 3.64; N 6.32.

(*E*)-4-(4-bromobenzylidene)-3-methylisoxazol-5(4*H*)-one (4c):Yellow colored liquid,obtained from 4-bromobenzaldehyde (0.185g, 1 mmol, 1equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum, (KBr) ν: 1730, 1531, 1216, 1120, 870, 773 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.4 (s, 3H, CH_3), 7.3 (s, 1H, vinyl), 7.70-7.72 (d, 2H, ArH, *J* = 8.0), 7.91-7.93 (d, 2H, ArH, *J* = 8.0); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.2, 122.2, 125.3, 131.5, 131.8, 134.6, 150.9, 164, 175.1. MS, m/z: 264.97 (M^+), 266.97, 265.98, 267.98, 266.98, 268.98. Found, %: C 49.66; H 3.05; N 5.22. $C_{11}H_8BrNO_2$. Calculated, %: C 49.65; H 3.03; N 5.26.

(*E*)-4-(4-methoxybenzylidene)-3-methylisoxazol-5(4*H*)-one (4d): Yellow solid, M.p:174-175°C, obtained from 4-methoxybenzaldehyde (0.136 g, 1 mmol, 1equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum, (KBr) ν: 1758, 1610, 1269, 1038, 889, 770 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.41 (s, 3H, CH_3), 3.88 (s, 3H, OCH_3), 6.98-7.00 (d, 2H, ArH, *J* = 8.8), 7.40 (s, 1H, vinyl), 7.92-7.94 (d, 2H, ArH, *J* = 8.8); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.0, 56.1, 114.6, 125.3, 130.2, 150.1, 162.1, 164.1, 175.3. MS, m/z: 217.01(M+), 200.23, 159.13, 110.08, 89.02. Found, %: C 66.34; H 5.11; N 6.43. $C_{12}H_{11}NO_3$. Calculated, %: C 66.35; H 5.10; N 6.45.

(*E*)-4-(4,5-dimethoxybenzylidene)-3-methylisoxazol-5(4*H*)-one (4e): Amorphous solid, obtained from 3,4-dimethoxybenzaldehyde (0.166 g, 1 mmol, 1equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum, (KBr) ν: 1756, 1602, 1268, 1038, 887, 766 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.41 (s,

3H, CH_3), 3.88 (s, 6H, $2OCH_3$), 6.81-7.30 (m, 3H, Ar-H), 7.40 (s, 1H, vinyl); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.3, 56.4, 111.5, 111.7, 122.8, 125.7, 128.9, 149.0, 149.7, 150.9, 164.6, 175.0. MS, m/z: 247.08 (M^+), 248.09, 249.09. Found, %: C 67.53; H 5.66; N 6.07. $C_{13}H_{13}O_4N$. Calculated, %: C 67.52; H 5.67; N 6.06.

(*E*)-4-(3,4,5,-trimethoxybenzylidene)-3-methylisoxazol-5(4*H*)-one (4f): Amorphous solid, obtained from 3,4,5-trimethoxybenzaldehyde (0.196 g, 1 mmol, 1equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv). IR spectrum, (KBr) ν: 1751, 1589, 1265, 1038, 881, 730 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.40 (s, 3H, CH_3), 3.89 (s, 9H, $3OCH_3$), 7.13-7.15 (d, 2H, ArH, *J* = 8.8), 7.40 (s, 1H, vinyl); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.2, 56.3, 60.5, 103.9, 125.4, 130, 138.5, 150.8, 153.0, 164.8, 174.3. MS, m/z: 277.10 (M^+), 278.10, 279.10.Found, %: C 60.67; H 5.41; N 5.02. $C_{14}H_{15}NO_5$. Calculated, %: C 60.64; H 5.45; N 5.05.

(*E*)-4-(4-nitrobenzylidene)-3-methylisoxazol-5(4*H*)-one (4g): Amorphous solid, obtained from 4-nitrobenzaldehyde (0.151 g, 1 mmol, 1equiv), ethyl acetoacetate (0.390 g, 3 mmol, 3 equiv), hydroxylamine hydrochloride (0.069 g, 1 mmol, 1 equiv) and zinc chloride (1.2 equiv), IR spectrum, (KBr) ν: 1748, 1620, 1550, 1052, 703 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.2 (s, 3H, CH_3), 6.99-7.01 (d, 2H, ArH, *J* = 8.8), 7.4 (s, 1H, vinyl), 7.93-7.94 (d, 2H, ArH, *J* = 8.8); ^{13}C NMR (400 MHz, $CDCl_3$): δ 17.3, 123.9, 125.1, 132.2, 139.0, 147.1, 150.7, 164.7, 174.6. MS, m/z: 232.05 (M^+), 233.05. Found, %: C 56.91; H 3.45; N 12.35. $C_{11}H_8N_2O_4$. Calculated, %: C 56.90; H 3.47; N 12.06.

Anticancer activity

MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] assay for cytotoxicity: MTT assay was performed as described previously [52]. Human breast adenocarcinoma-MCF7 cells ($2x10^3$) were scattered in each well of a 96-well plate and were permitted to adhere and spread for 24 hrs. Afterwards, cells were treated with different concentrations (0.1, 1.0, 10.0 and 100 µg mL^{-1}) of test compounds prepared in 10% dimethyl sulphoxide (DMSO) and incubated at 37°C for 24 h. *Doxorubicin* was taken as a positive control and first column containing no drug taken as negative control. After a period of 24 hr, added MTT solution (10 µL of 10 mg mL^{-1}) to each cultured well and further incubated for 4 hr to allow the formation of formazan crystals. And again added 100 µL of MTT solution and incubation continued for 12 h. The absorbance value was measured in each well at 570 nm with the help of an ELISA plate reader (ELx800, Biotech, VT, USA) and matched with untreated control. Finally cell viability of was calculated using the following formula:

Cell viability % = (Absorbance of sample/Absorbance of control) x100

Further IC_{50} values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

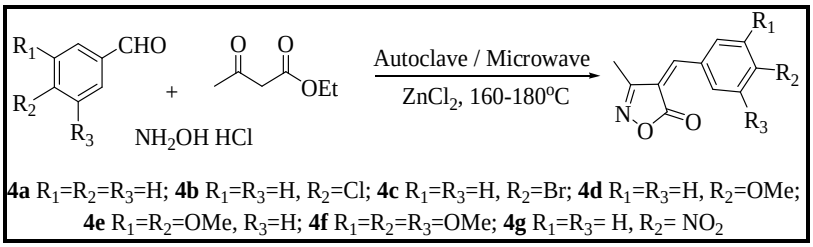
RESULTS AND DISCUSSION

X-ray structural study of the compound 4a: A single crystal of the compound **4a** with dimensions of 0.30 × 0.25 × 0.20 mm was chosen for X-ray diffraction studies. The crystallographic data were collected at 293K on a Bruker SMART APEX II X-ray diffractometer with graphite monochromated MoKα radiation (0.71073Å). Raw data was processed and reduced by using APEX2 and SAINT [54]. The crystal structure was solved by direct methods using SHELXS-97 [55, 56]. All non-hydrogen atoms were revealed in the first Fourier map itself. Anisotropic refinement of non-hydrogen atoms was started at this stage. Subsequent refinements were carried out with anisotropic thermal parameters for non-hydrogen atoms and isotropic temperature factors for the hydrogen atoms which were placed at chemically acceptable positions. Full-matrix least squares refinement was carried out using SHELXL-97 with a final residual values of R1=0.0419. The thermal ellipsoid plot [57] at 50% probability of the molecule is shown in Figure 1. In our previous paper [58], we have reported the

experimental and crystallographic data of compound **4a**. The complete crystallographic data set was deposited at the Cambridge Crystallographic Data Center (CCDC 909795).

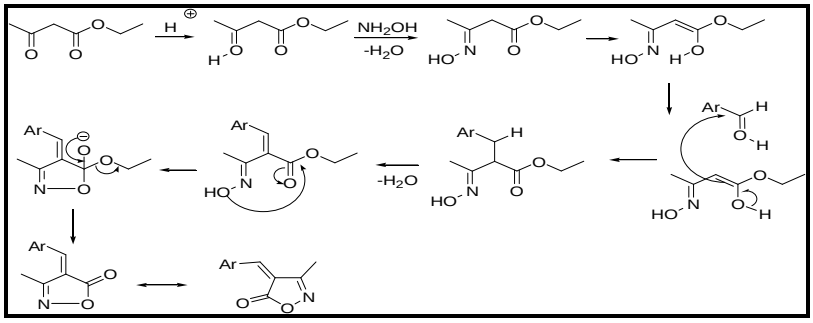
Now a day's demand for drug rapidly increases due to lack of curable properties, side effects and solvent effects in newly synthesized derivatives. In order to tailor the desired moiety, which is more selective towards pharmaceutical field we were excited to synthesize our title compound without using any solvent. In continuation of our research work in autoclave reaction [59], here we have prepared some derivatives of (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-one by novel ways i.e., autoclave and microwave assisted reaction using benzaldehyde derivatives and ethyl acetoacetate in the presence of hydroxylamine hydrochloride and anhydrous zinc chloride as precursors without using any solvent. These two novel methods has received vital advertence to prepare title compound due to their lineament such as clean, green, less time consuming and paramount yield.

The formation of **4a** (Scheme 1) follows intramolecular cyclization followed by the liberation of water molecules. Initially the reaction starts with the protonation of ethyl acetoacetate oxygen from hydroxyl amine hydrochloride, which further releases water molecule and forms ethyl 3-(hydroxyimino) butanoate. In the next step, Knoevenagel reactions between aromatic aldehydes and ethyl 3-(hydroxyimino) butanoate through intramolecular cyclization yields the desired product. Here zinc chloride acts as a mild catalyst [60] dehydrates the water molecules liberated and accelerates the rate of reaction (Scheme 2).



4a R_1=R_2=R_3=H; **4b** R_1=R_3=H, R_2=Cl; **4c** R_1=R_3=H, R_2=Br; **4d** R_1=R_3=H, R_2=OMe; **4e** R_1=R_2=OMe, R_3=H; **4f** R_1=R_2=R_3=OMe; **4g** R_1=R_3= H, R_2= NO_2

Scheme 1–Synthesis of compounds **4a-g**



Scheme 2. Possible mechanism for the formation of compounds **4a-g.**

The structural analysis of our title compounds were derived by their spectral data. A sharp singlet at δ 2.40 ppm in the spectra of ^{1}H NMR of **4a** showed the presence of methyl group (–CH_3) and in addition to this, a singlet at δ = 7.40 ppm and multiplet at δ = 7.50-7.56 ppm revealed the presence of vinyl group and benzene ring respectively. For halogenated derivatives there is a small increase in δ value, i.e., δ 7.57 ppm for chloro, and δ 7.70 ppm for bromo derivatives due to shielding effect of halogens at para position. When comes to methoxy substituted derivatives appearance of extra singlet at δ= 3.88 indicates the presence of -OCH_3 group. In ^{13}C NMR spectrum we have observed the characteristic signals at 17.1 ppm and 176.0 ppm due to CH_3 and C=O of the isoxazole ring. The mass spectra show intense peak at 187.04 (M^+) confirms the formation of cyclized product. In addition to this, a single crystal X-ray structural study further confirms the structure of compound **4a** [55] (Figure 1).

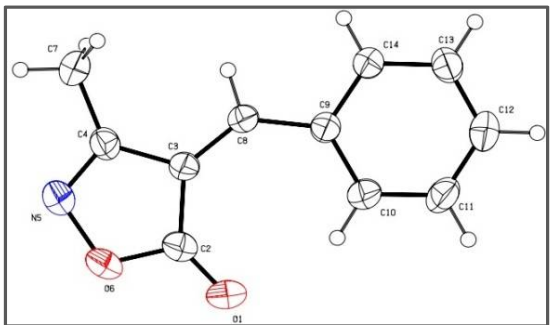


Figure 1. Perspective diagram of the molecule **4a** with 50% probability displacement ellipsoids.

With addition to this, the product yield was enhanced by varying the quantity of ethyl acetoacetate with respect to benzaldehyde derivatives (Table 1). Also, autoclave fusion reaction in the absence of solvent (yields; 72-95%) was better than the microwave solvent free method (61-81%). Since the product yield obtained at 1:3 ratio of benzaldehyde and ethyl acetoacetate was better, we performed all the reactions by taking 1:3 ratios of precursors. Finally the product was evidenced by spectroscopic techniques such as IR, ^{1}H NMR, ^{13}C NMR, Mass, elemental analysis and also by single crystal X-ray studies.

Table 1. Comparison of autoclave and microwave reaction conditions for the synthesis of compounds **4a-g**.

Entry	Ratio	Reaction time		Yield (%)	
	Benzaldehyde : Ethyl acetoacetate	Autoclave (h)	Microwave (min)	Autoclave	Microwave
4a	1 : 1	2	20	91	70
	1 : 2	2	20	92	72
	1 : 3	2	20	95	79
4b	1 : 1	2	20	90	78
	1 : 2	2	20	91	75
	1 : 3	2	20	94	80
4c	1 : 1	2	20	89	71
	1 : 2	2	20	94	77
	1 : 3	2	20	95	81
4d	1 : 1	2	20	87	73
	1 : 2	2	20	89	76
	1 : 3	2	20	94	78
4e	1 : 1	2	20	81	70
	1 : 2	2	20	83	74
	1 : 3	2	20	88	76
4f	1 : 1	2	20	91	63
	1 : 2	2	20	93	68
	1 : 3	2	20	94	76
4g	1 : 1	2	20	72	61
	1 : 2	2	20	74	62
	1 : 3	2	20	74	63

In summary we reported a benign, green and solvent free protocol to tailor (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-ones without using any solvents in the presence of zinc chloride as mild catalyst by both autoclave and microwave reactions. And also present work mainly concentrated to design a potent molecule by green and eco-friendly reaction medium along with shorter reaction time and easy work up.

APPLICATION

Anticancer activity of compounds 4a to 4g: The MTT cell proliferation assay has been widely accepted as a reliable way to measure the cell proliferation rate and conversely when metabolic events lead to apoptosis or necrosis. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. The synthesized compounds **4(a-g)** possesses countable cytotoxic effects. Even though they possesses different derivatives their cytotoxic effects remains same without much variations. In general, the compounds **4b**, **4c** and **4d**which have*p*-chloro, *p*-bromo and *p*-methoxygroups respectively, weremore potent than**4a**, **4e**, **4f** and **4g**. Amongst**4b**, **4c**and **4d**, the compound **4b** has shown better activity. The results were reported as percentage survival of the cells when compared to that of the untreated control cells ± standard deviation in table 2. The IC_{50} for the standard drug *Doxorubicin* (DOX) was found to be 20 µg mL^{-1}.

Table 2. MTT assay of the compounds **4(a-g)**

Products	Vehicle Control	10 µg mL^{-1}	50 µg mL^{-1}	100 µg mL^{-1}	IC_{50} µg mL^{-1}
4a	100 ± 3.90	96.2 ± 4.40	80.8 ± 5.21	59.2 ±3.25	60
4b	100 ± 7.03	80.2 ± 5.23	49.6 ± 2.32	41.6 ± 6.23	35
4c	100 ± 4.20	81.6 ± 2.36	53.4 ± 1.23	42.4 ± 2.36	45
4d	100 ± 3.29	81.5 ± 6.32	50.2 ± 3.23	38.1 ± 2.36	40
4e	100 ± 5.00	91.4 ± 2.32	76.1 ± 1.23	60.6 ±2.36	70
4f	100 ± 4.90	90.9 ± 2.36	75.1 ± 5.36	64.1 ± 5.62	70
4g	100 ± 7.00	95.4 ± 2.36	71.5 ± 1.84	59.6 ± 4.36	65
DOX	---	---	---	---	20

CONCLUSION

Here we reported a benign green and solvent free protocol to tailor (*E*)-4-benzylidene-3-methylisoxazol-5(4*H*)-ones without using any solvents in the presence of zinc chloride as catalyst by both autoclave and microwave reactions. The protocol helps in designing a potent molecule with shorter reaction time and easy work up also.

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