Synthesis of 2-Oxo/Thioxooctahydroquinazolin-5-one Derivatives and Their Evaluation as Anticancer Agents

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An environment friendly method for the synthesis of 2-oxo/thioxooctahydroquinazolin-5-one derivatives has been devised using Ceric ammonium nitrate (CAN) as catalyst and polyethylene glycol (PEG) as solvent. The cytotoxic effect of these compounds was studied on U87 human glioma cells, compounds 4c, 4d and 4e are found to exhibit excellent activity at a concentration as low as 0.06 µg/ml.

Key words Ceric ammonium nitrate; polyethylene glycol; U87 human glioma cell; 2-oxo/thioxooctahydroquinazolin-5-one

Cancer, a disease of worldwide importance, according to the American Cancer Society, is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. It is a fatal disease that has posed serious threat to human health. Therefore, identification of novel potent and selective anticancer drugs remains one of the most pressing health problems. Quinazolines are very interesting heterocycles, which are ubiquitous in nature and show a wide range of biological activities.^{1–8)} Several promising anticancer active derivatives are found to contain quinazolines such as raltitrexed and thymitaq.^{9–11)} Despite their biological activities, no recent progress on their syntheses has been made.

The present study aimed to devise a novel and environment friendly method for the synthesis of some quinazoline derivatives and evaluation of their biological activity as potential anticancer agents.

Various methods have been reported in literature for the synthesis of derivatives of quinazolines, the oldest being the Biginelli synthesis.¹²⁾ Other methods involve a refluxing solvent like benzene or xylene with azeotropic water removal,^{13,14)} refluxing in ethanol acetic acid mixtures^{15,16)} and by reaction in alkali media. Quinazoline derivatives are also synthesized starting from anthranilic acid,^{17–22)} benzoni-trile¹⁹⁾ and so on with an appropriate substituent to have specific functionality and activity. But most of these methods

have resorted to harsh conditions, low yields, longer reaction times and use of expensive and hazardous chemicals with side product formation. Furthermore these methodologies do not meet the requirement of green chemistry.

Development of a synthetic protocol that is devoid of the above mentioned problems is nature friendly, simple, efficient and cost effective remains an ever challenging objective.

Recently polyethylene glycol (PEG) is found to be an interesting solvent system for organic synthesis.^{23–25)} PEG and its monomethyl ethers are inexpensive, thermally stable, recoverable and non toxic media for phase transfer catalysis.^{26,27)}

Ceric(IV) ammonium nitrate (CAN) has emerged as an important reagent for the construction of carbon–carbon and carbon–heteroatom bonds. In addition, many advantages such as excellent solubility in water and easy work up procedure make CAN a potent catalyst in organic synthesis. Besides this, CAN is able to catalyze various organic transformations not only based on its electron transfer capacity but also with its Lewis acidic property.^{28–30)}

The environmentally benign nature of PEG and the versatility of CAN encouraged us to couple them together and study their utility in the synthesis of 2-oxo/thioxooctahydroquinazolin-5-one derivatives (Charts 1, 2).

Chart 1. Synthesis of 2-Oxo/Thioxooctahydroquinazolin-5-one

Chart 2. Synthesis of 2-Oxo/Thioxo-7,7-dimethyloctahydroquinazolin-5-one

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Results and Discussion

Chemistry In an initial endeavor the reaction between cyclohexadione (1 mmol), benzaldehyde (1 mmol) and urea (1 mmol) was performed in traditional organic solvent (EtOH, Table 1, entry 1). The reaction mixture was stirred for 6 h at 50 °C to obtain 60% of octahydroquinazolin-2,5-dione **4a**, whereas the same reaction in the presence of PEG-400 as environmentally friendly medium provided 91% of **4a** (Table 1, entry 5) in only 1 h.

To further improve the yield and to optimize the reaction conditions, the same reaction was carried out in the presence

of 2 mol% of CAN under similar conditions. A tremendous improvement was observed and the yield of **4a** was increased upto 95% after stirring the mixture for only 30 min. With this optimistic result in hand, we further investigated the best reaction conditions by using different amounts of CAN. An increase in the quantity of CAN from 2 to 5 mol% not only decreased the reaction time from 30 to 20 min but also increased the product yield from 95 to 98%. This showed that the catalyst concentration plays a major role in optimization of the product yield. The use of 10 mol% of CAN decreased the yield of the product to 65%. A possible explanation for the low product yield is that the starting material or the product may have been destroyed during the reaction when excess amount (10 mol%) of CAN was used in the reaction and thus 5 mol% CAN is the suitable choice for the optimum yield of 2-oxo/thioxooctahydroquinazolin-5-one (Table 2).

The effect of temperature on reaction rate as well as yields of products was also investigated. Faster reactions occurred on increasing the temperature but the product yield decreased at high temperature because one of the reactants oxidizes at high temperature in presence of CAN (Table 3).

In order to extend the range of substrates, we intended to apply our methodology to a wide range of aldehydes in the presence of 5 mol% CAN under similar conditions and expected satisfactory results were obtained (Table 4).

Table 1. Synthesis of Various 2-Oxo/Thioxooctahydroquinazolin-5-one in Various Solvents^{a)}

Entry	Solvent	Time (h)	Yield ^{b)} (%)
1	Ethanol	6	60
2	Acetonitrile	6	62
3	Toluene	7	60
4	PEG 200	1	90
5	PEG 400	1	91
6	PEG 600	1	91

a) Reaction conditions: cyclohexadione (1 mmol), benzaldehyde (1 mmol), urea (1 mmol); temperature 50 °C. b) Isolated and unoptimised yields.

Table 2. Catalytic Activity Evaluation for Synthesis of 2-Oxo/Thioxooctahydroquinazolin-5-one^{a)}

Entry	CAN (mol%)	Time (min)	Yield ^{b)} (%)
1	0	60	91
2	2	30	98
3	5	20	98
4	10	15	67

a) Reaction conditions: cyclohexadione (1 mmol), benzaldehyde (1 mmol), urea (1 mmol); solvent PEG 400; 50 °C. b) Isolated and unoptimised yields.

Table 3. Effect of Temperature^{a)}

Entry	Temperature (°C)	Time (min)	Yield ^{b)} (%)
1	50	20	98
2	65	15	98
3	80	12	93
4	100	10	78

a) Reaction conditions: cyclohexadione (1 mmol), benzaldehyde (1 mmol), urea (1 mmol); solvent PEG 400; CAN (5 mol%). b) Isolated and unoptimised yields.

Table 4. Synthesis of Various 2-Oxo/Thioxooctahydroquinazolin-5-one Using CAN and PEG^{a)}

Product	Active methylene compd.	R	X	mp (°C)	Time (min)	Yield ^{b)} (%)
4a		-C ₆ H ₅	O	308—310	15	98
4b		-4OCH ₃ C ₆ H ₄	O	284—285	20	98
4c		-CH ₂ CH ₃	S	179—181	20	98
4d		-C ₃ H ₈	O	250—253	25	92
4e			S	231—234	20	97
6a		C ₆ H ₅	O	289—291	15	98
6b		-4OCH ₃ C ₆ H ₄	O	298—299	20	96
6c		-CH ₂ CH ₃	S	167—171	20	92
6d		-C ₃ H ₈	O	231—233	25	92
6e			S	208—210	20	97

a) Reaction conditions: active methylene compound (1 mmol), aldehyde (1 mmol), urea/thiourea (1 mmol), CAN (5 mol%); solvent PEG 400; temperature 50 °C. b) Isolated and unoptimised yields.

Table 5. Recycling Yields

No. of cycles ^{a)}	Fresh	Run 1	Run 2	Run 3
Yield ^{b)} (%)	98	98	97	98
Time (min)	20	20	20	20

a) Reaction conditions: cyclohexadione (1 mmol), benzaldehyde (1 mmol), urea (1 mmol), CAN (5 mol%); solvent PEG 400; temperature 50 °C.

Table 6. Viability at Concentration of 310, 150, 78, 39 and 2 in $\mu\text{g/ml}$ and IC_{50} of **4a–e** and **6a–e**

Compds. Concentration ($\mu\text{g/ml}$)	Viability (%)					IC_{50} $\mu\text{g/ml}$
	310	150	78	39	2	
4a	8.241	7.234	50	100	100	0.091
4b	7.509	8.15	16.57	100	100	0.073
4c	6.318	6.501	14.8	99.7	94.597	0.069
4d	6.959	7.142	13.278	100	100	0.067
4e	6.227	5.952	20.238	95.23	90.018	0.068
6a	10.256	9.249	45.238	100	100	0.112
6b	8.764	11.78	75.659	100	100	0.141
6c	8.462	6.85	37.779	100	100	0.100
6d	8.966	7.555	39.19	65.109	87.346	0.085
6e	10.376	14.91	61.152	88.555	87.447	0.121

To check the ecofriendliness of PEG, we recycled PEG 400 for several times (Table 5). The reaction proceeded cleanly with consistent results, although a weight loss of ca. 5% of PEG 400 was observed from cycle to cycle due to mechanical loss.

Pharmacology The cytotoxic effects of compounds **4a–e** and **6a–e** on U87 human glioma cells was studied. The efficacy of the test compound was compared with control on the basis of % cytotoxicity. The % cytotoxicity for the compounds at five different concentrations was calculated as:

$$\% \text{ cytotoxicity} = (100 - \text{test optical density (OD)} / \text{control OD}) \times 100$$

It was evident from the study that all the 2-oxo/thioxooctahydroquinazolin-5-one derivatives had cytotoxic effect on U87 human glioma cells.

The IC_{50} values for all the compounds was calculated and tabulated. The IC_{50} values for compounds **4c**, **4d** and **4e** was found to be similar and lowest amongst all others. Amongst all the 4-aryl-2-oxo/thioxooctahydroquinazolin-5-one derivatives, **4d** was found to be the most effective and **6b**, the least effective (Table 6).

Conclusion

In conclusion we have developed an effective catalytic system PEG 400/CAN for the synthesis of 2-oxo/thioxooctahydroquinazolin-5-one as potential anticancer agents. The methodology is simple, efficient and environment friendly with simple work up. We could reuse our solvent system several times. All these characteristics of our protocol make the reaction quite suitable for scale up and commercialization.

Experimental

Chemistry. General All chemicals were purchased from Sigma-Aldrich and were used as such. All reactions and the purity of 2-oxo/thioxooctahydroquinazolin-5-one were monitored by thin layer chromatography (TLC) using aluminium plates coated with silica gel (Merck) using 15% ethyl acetate, 5% methanol and 80% petroleum ether as an eluent. The isolated

products were further purified by column chromatography using silica gel (Sigma-Aldrich 24, 217-9, 70, 35–70, mesh 40 Ao surface area 675 m^2/g) and the purified products were recrystallized. IR spectra were recorded on a Perkin-Elmer FTIR-1710 spectrophotometer using Nujol film. $^1\text{H-NMR}$ spectra were recorded on a Bruker Avance spectropspin 300 (300 MHz) using TMS as an internal standard and chemical shifts are in δ . GC-MS mass spectra were recorded on a waters LCT Micromass. The temperature of the reaction mixture was measured through a non-contact infrared thermometer (AZ, Mini Gun type, Model 8868).

General Procedure for the Synthesis of 2-Oxo/Thioxooctahydroquinazolin-5-one In a 50 ml round bottom flask, cyclohexadione (1 mmol), aromatic aldehyde (1 mmol) and urea/thiourea (1 mmol) in PEG (0.2 ml) were mixed and stirred at room temperature. To this CAN (Ceric ammonium nitrate) (5 mol%) was added. The progress of the reaction mixture was monitored by TLC. After completion of the reaction, the reaction mixture was cooled with a dry ice-acetone bath to precipitate the PEG and extracted with ether (PEG being insoluble in ether). The ether layer was decanted, dried and concentrated under reduced pressure. The product though seen as a single compound by TLC, was subjected to further purification by silica gel column chromatography using 20% ethyl acetate and 80% hexane as an eluent to yield the products **4a–e** and **6a–e**. The recovered PEG can be reused for consecutive runs. The structures of all the products were unambiguously established on the basis of their spectral analysis (IR, $^1\text{H-NMR}$ and mass spectral data). The spectral data for the new products are listed.

Spectral Data 4-Phenyl-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**4a**): IR (KBr): 3320 (NH), 1457 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 1.10–2.72 (m, 6H, CH_2), 5.15 (s, 1H), 7.15–7.69 (m, 5H, Ar), 8.2 (s, 1H, NH), 10.7 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 17.3, 31.3, 46.7, 48.9, 115.5, 125.7, 126.72, 128.1, 148.0, 159.3, 190.4. MS (electron ionization (EI)) m/z : Calcd for $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_2$: 242.1055; Found: 242.1121. *Anal.* Calcd for $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_2$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.56; H, 5.97; N, 11.41.

4-(4-Methoxyphenyl)-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**4b**): IR (KBr): 3418 (NH), 1421 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 1.12–2.56 (m, 6H, CH_2), 3.63 (s, 3H, OCH_3), 5.27 (s, 1H), 7.17–7.36 (m, 4H, Ar), 7.90 (s, 1H, NH), 10.81 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 18.9, 36.3, 42.4, 54.2, 57.5, 115.1, 117.2, 126.7, 139.2, 158.7, 182.3, 192.4. MS (EI) m/z : Calcd for $\text{C}_{15}\text{H}_{16}\text{N}_2\text{O}_3$: 272.1161; Found: 272.1211. *Anal.* Calcd for $\text{C}_{15}\text{H}_{16}\text{N}_2\text{O}_3$: C, 66.16; H, 5.92; N, 10.29. Found: C, 66.29; H, 5.78; N, 10.37.

4-Ethyl-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**4c**): IR (KBr): 3320 (NH), 1457 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 0.92 (t, 3H, CH_3), 1.27–2.89 (m, 8H, CH_2), 4.12 (s, 1H), 7.92 (s, 1H, NH), 10.2 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 10.2, 18.7, 27.6, 37.4, 42.3, 53.8, 115.5, 138.0, 159.7, 192.4. MS (EI) m/z : Calcd for $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_2$: 194.1055; Found: 194.1120. *Anal.* Calcd for $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_2$: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.69; H, 7.31; N, 14.54.

4-Propyl-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**4d**): IR (KBr): 3334 (NH), 1452 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 0.93 (t, 3H, CH_3), 1.32–2.73 (m, 10H, CH_2), 4.17 (s, 1H), 8.0 (s, 1H, NH), 10.2 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 15.3, 17.2, 18.7, 38.4, 39.1, 42.8, 127.1, 143.2, 156.3, 193.6. MS (EI) m/z : Calcd for $\text{C}_{11}\text{H}_{16}\text{N}_2\text{O}_2$: 208.1212; Found: 208.1178. *Anal.* Calcd for $\text{C}_{11}\text{H}_{16}\text{N}_2\text{O}_2$: C, 63.44; H, 7.74; N, 13.45. Found: C, 63.57; H, 7.62; N, 13.39.

4-(Benzo[1,3]dioxol-5-yl)-2-thioxo-2,3,4,6,7,8-hexahydro-1*H*-quinazolin-5-one (**4e**): IR (KBr): 3432 (NH), 1467 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 1.10–2.74 (m, 6H, CH_2), 5.12 (s, 1H), 5.78 (s, 2H, CH_2), 6.66–6.78 (m, 3H, Ar), 8.01 (s, 1H, NH), 10.53 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 18.7, 35.3, 42.9, 93.7, 113.5, 117.6, 118.9, 121.3, 136.5, 145.8, 146.4, 156.5, 178.3, 194.6. MS (EI) m/z : Calcd for $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_3\text{S}$: 302.0725; Found: 302.0812. *Anal.* Calcd for $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_3\text{S}$: C, 59.59; H, 4.67; N, 9.27. Found: C, 59.71; H, 4.41; N, 9.47.

7,7-Dimethyl-4-phenyl-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**6a**): IR (KBr): 3327 (NH), 1462 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 0.98 (s, 6H, CH_3), 2.08–2.91 (m, 4H, CH_2), 5.01 (s, 1H), 7.10–7.27 (m, 5H, Ar), 8.2 (s, 1H, NH), 10.6 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) δ : 17.1, 26.6, 48.2, 49.1, 54.3, 115.7, 122.5, 123.1, 125.9, 144.4, 146.9, 161.2, 192.8. MS (EI) m/z : Calcd for $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_2$: 270.1368; Found: 270.1319. *Anal.* Calcd for $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.18; H, 6.86; N, 10.29.

4-(4-Methoxyphenyl)-7,7-dimethyl-4,6,7,8-tetrahydro-1*H*,3*H*-quinazolin-2,5-dione (**6b**): IR (KBr): 3418 (NH), 1463 ($\text{C}=\text{C}$) cm^{-1} . $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ : 0.98 (s, 6H, CH_3), 2.18–2.89 (m, 4H, CH_2), 3.16 (s, 3H, OCH_3), 5.05 (s, 1H), 6.19–7.17 (m, 4H, Ar), 8.2 (s, 1H, NH), 10.1 (s,

1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ: 17.6, 27.3, 46.9, 56.5, 57.0, 57.7, 112.1, 115.5, 128.1, 138.0, 156.3, 160.4, 179.3, 190.4. MS (EI) *m/z*: Calcd for C₁₇H₂₀N₂O₃: 300.1474; Found: 300.1317. *Anal.* Calcd for C₁₇H₂₀N₂O₃: C, 67.98; H, 6.71; N, 9.33. Found: C, 67.72; H, 6.59; N, 9.42.

4-Ethyl-7,7-dimethyl-2-thioxo-2,3,4,6,7,8-hexahydro-1H-quinazolin-5-one (**6c**): IR (KBr): 3448 (NH), 1448 (C=C) cm⁻¹. ¹H-NMR (300 MHz, CDCl₃) δ: 0.91 (t, 3H, CH₃), 1.0 (s, 6H, CH₃), 1.59–2.72 (m, 6H, CH₂), 4.42 (s, 1H), 8.1 (s, 1H, NH), 10.7 (s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ: 10.1, 17.8, 26.7, 26.9, 46.4, 47.2, 54.8, 115.1, 143.2, 157.6, 196.9. MS (EI) *m/z*: Calcd for C₁₂H₁₈N₂O₂S: 238.1140; Found: 238.1321. *Anal.* Calcd for C₁₂H₁₈N₂O₂S: C, 60.47; H, 7.61; N, 11.75. Found: C, 60.54; H, 7.42; N, 11.59.

7,7-Dimethyl-4-propyl-4,6,7,8-tetrahydro-1H,3H-quinazolin-2,5-dione (**6d**): IR (KBr): 3350 (NH), 1465 (C=C) cm⁻¹. ¹H-NMR (300 MHz, CDCl₃) δ: 0.91 (t, 3H, CH₃), 1.21 (s, 6H, CH₃), 1.78–2.78 (m, 8H, CH₂), 4.48 (s, 1H), 8.2 (s, 1H, NH), 10.1 (s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ: 15.7, 17.1, 17.8, 27.9, 38.5, 45.1, 47.3, 54.5, 120.8, 143.8, 156.2, 198.7. MS (EI) *m/z*: Calcd for C₁₃H₂₀N₂O₂: 236.1525; Found: 236.1329. *Anal.* Calcd for C₁₃H₂₀N₂O₂: C, 66.07; H, 8.53; N, 11.85. Found: C, 66.19; H, 8.42; N, 11.56.

4-(Benzo[1,3]dioxol-5-yl)-7,7-dimethyl-2-thioxo-2,3,4,6,7,8-hexahydro-1H-quinazolin-5-one (**6e**): IR (KBr): 3439 (NH), 1455 (C=C) cm⁻¹. ¹H-NMR (300 MHz, CDCl₃) δ: 1.07 (s, 6H, CH₃), 2.17–2.48 (m, 4H, CH₂), 5.12 (s, 1H), 5.93 (s, 2H, CH₂), 6.67–6.92 (m, 3H, Ar), 8.01 (s, 1H, NH), 10.52 (s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ: 17.4, 27.2, 48.7, 53.4, 56.2, 93.7, 112.3, 114.6, 115.1, 120.8, 136.2, 143.5, 145.4, 156.7, 176.3, 192.7. MS (EI) *m/z*: Calcd for C₁₇H₁₈N₂O₃S: 330.1038; Found: 330.1074. *Anal.* Calcd for C₁₇H₁₈N₂O₃S: C, 61.80; H, 5.49; N, 8.48. Found: C, 61.64; H, 5.73; N, 8.70.

Pharmacology. Cell Culture U87 human glioma cells and Human Embryonic Kidney 293 (HEK-293) cells were obtained from the Department of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Defense Research and Development Organization, Delhi, India. U87 human glioma cells were cultured in low glucose (1 g/l) DMEM (Dulbecco's modified Eagle's medium, Himedia, India) supplemented with 10% fetal bovine serum and a mixture of penicillin (100 U/ml) and streptomycin (50 μg/ml) of medium, under a humidified 5% CO₂ atmosphere at 37 °C. Cells were cultured to approximately 50% confluence at 37 °C with 5% CO₂ overnight to insure complete attachment of cells to the culture matrix. The next day, cells were treated with or without compounds.

In Vitro Cytotoxicity Assay 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay³¹ was performed in order to examine the cytotoxic effects of the compounds on U87 cells. 5 × 10³ cells per well were seeded in 96 flat bottom well plates. The appropriate concentrations (μg/ml for U87 cells) of the compounds were then added to the cells. The cells were continuously treated for 24 h. The cytotoxicity was measured by adding 10 μl of 5 mg/ml of MTT (Sigma-Aldrich Inc., U.S.A.) to each well and incubated for another 2 h in CO₂ incubator. The purple formazan crystals were dissolved by adding 100 μl of dimethyl sulfoxide (DMSO) to each well. The absorbance was read at 570 nm in a spectrophotometer (Biotek Synergy HT, U.S.A.). The cell death was calculated as follows:

$$\% \text{ cytotoxicity} = (100 - \text{test OD} / \text{control OD}) \times 100$$

The test result is expressed as the concentration of a test compound which inhibits the cell growth by 50% (IC₅₀).

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