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SYNTHESIS OF NOVEL 10-[3'- (N-SUBSTITUTED BUTYL)-3-CHLORO-4-FLUORO]-ACRIDONES TO TARGET HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC) has become a global health problem requiring a potent chemotherapeutic agent. Acridone and its derivatives are important anthracene analogue heterocyclic compounds as with high DNA affinity and interactive properties making it an important pharamocophore for designing several chemotherapeutic agents. Several 9-acridone derivatives with or without an alkyl side chain attached to the N-position have been found to exhibit antioxidant activities. So, several novel N^{10} substituted -4- Methyl acridones were synthesized and evaluated for its anticancer activity in HepG2 cell lines and its correlation with antioxidant scavenging property. Results suggested that, all the synthesized compounds have a potent cytotoxic activity with a significantly less IC_{50} as compared to the standard sorafenib.

KEYWORDS: Hepatocellular carcinoma; Acridone; HepG2 cell line; Anticancer; antioxidant; Sorafenib.

INTRODUCTION

Hepatoccellular carcinoma (HCC) is a global health problem with approximately around 8 lakh death per year, moreover there exist a very limited treatment options.[1,2] Surgery remains to be the most effective way for the suppression of HCC but it is associated with sever other problem of relapse and distant organ invasion after surgery.[3] So to get rid of such invasion and relapse a chemotherapeutic agent is highly recommended that can clear the survived tumor cells post dissection. Therefore, choosing appropriate anti-tumor agents and planning their administration is an indispensable part of systemic treatment HCC. Acridone derivatives are one such novel chemotherapeutic agent that can be explored for the treatment of HCC.

Acridone is a biologically active fused heterocyclic rings containing compound known to be associate with several biological activities. It has carbonyl group at 9^{th} position and nitrogen at 10^{th} position and is an oxidized product of acridine. Acridone is also known by the name of 9(10H)-acridinone, acridine-9-one, 9-acridanone, acridine 9, 10-dihydro-9-oxo, 9, 10-dihydro-9-oxo acridine, acridinone and 9-azanthracene-10-one.[5,6] Its derivatives are known for its chemotherapeutic agents that were initially antibacterial and antiparastic agents.[7,8] The biology of acridines is mainly attributed to the planarity of these aromatic structures which can intercalate within the double –stranded DNA structure, thus interfering with nuclear machinery.[9] In view of the above facts, it is of considerable interest to synthesize the titled compounds with a hope to obtain potent anticancer agents. A survey of chemical literature has discovered that the acridone ring nucleus substituted at N^{10} position with tertiary amino groups (N-methylpiperazino, piperidino, morpholidino, diethylamino, diethanolamino and (β – hydroxyethyl) piperizine) at a distance of 3 to 4 carbon reported to posses anticancer activity.[10,11] Moreover compound with anticancer agents are reported to have a potent antioxidant property.[12,13] With an intention of discovering a better anticancer agent and antioxidant a series of possible compounds have been prepared on, N^{10} acridones by Ulmann's condensation method.

MATERIALS AND METHODS

Synthesis

Preparation of 3-Chloro-4-Fluoro- Diphenylamine-2-Carboxylic acid

To a mixture of o-chlorobenzoic acid (A) (5g, 0.032 mmole), 0-3 chloro, 4-fluoro aniline (5g 0.046 mmoles) and copper powder (0.2 g) in 30ml isoamyl alcohol, dry potassium carbonate (10 g) was slowly added and the contents were allowed to reflux for 6 hours on an oil bath. Precipitate formed was filtered, washed with hot water and collected. The crude acid was dissolved in aqueous Sodium hydroxide solution, boiled in the presence of activated charcoal and filtered. On acidification of the filtrate with concentrated hydrochloride acid, light Yellowish precipitate was obtained which was washed with hot water and recrystallized from ethanol to give light yellow solid (yield 72%, mp 188 0C). IR Spectrum (cm-1) 3055

(OH), 2420 (NH), 1639 (C=O), 1097 (C-F), and 846 (C-Cl).

Cyclisation of 3-Chloro-4-Flouro-diphenylamine to 3-Chloro-4- Fluoro Acridone

Six grams of amino benzoic acid was taken in a round bottom flask to which was added 60 g of polyphosphoric acid. Shaken well and heated on a water bath at 100^0C for 3 hours. Appearance of yellow colour indicated the completion of the reaction. Then, it was poured into one liter of hot water and made alkaline by liquor ammonia. The yellow precipitate that formed was filtered, washed with hot water and collected. The sample of 3- Chloro, 4-Fluoro acridone (1) was recrystallized from acetic acid (yield 76%, mp 323^0C). Further, Purity of the compound was checked by TLC (chloroform: methanol = 24:1) and the purified product characterized by spectral methods. IR Spectrum (cm^{-1}) 2970 (NH), 1685 (C=O), 1087 (C-F), and 763(C-Cl). ^{1}H NMR ($CDCl_3$- d_6) δ: 10.8(S, 1H, NH), 7.2-8.6(m, 6H,Ar-H).

Synthesis of N^{10} - alkylated Acridones via Phase Transfer Catalysis

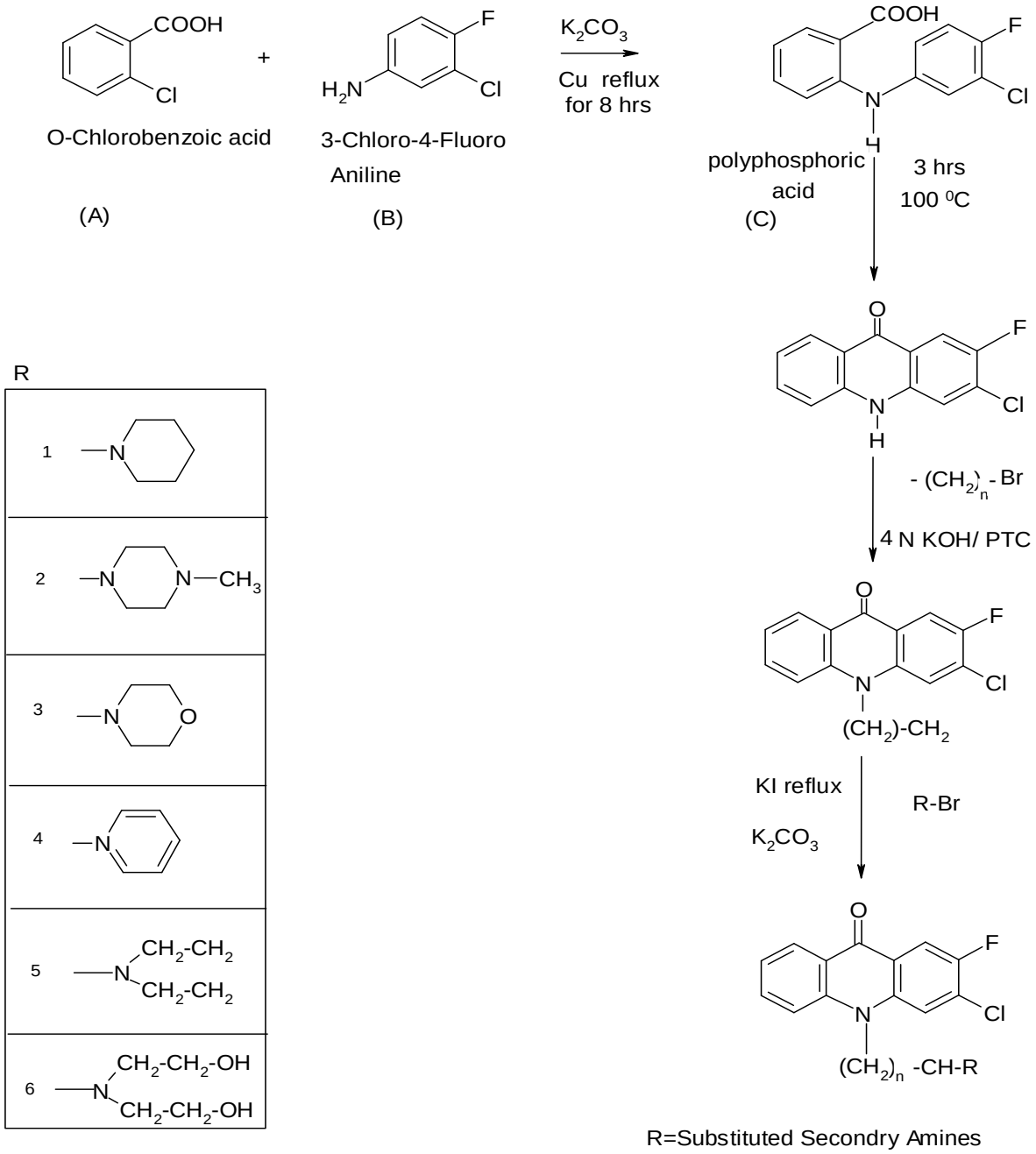
10-(3'-N-Chloropropyl] – 3-Chloro- 4 -Fluoro acridone

One gram (0.0047 mmole) of 3-Chloro-4- Flouro acridone was dissolved in 20ml tetrahydrofuran and added 25ml of 6N potassium hydroxide and 0.74g (2.30 mmole) tetrabutylammonium bromide to it. The reaction mixture was stirred at room temperature for half an hour and added 1- bromo -3-chlroprophane (0.69 mmole) slowly into the reaction mixture stirred for 24 hours at room temperature. Tetrahydrofuran was evaporated and the aqueous layer was extracted with chloroform. The chloroform layer was washed with water and organic layer dried over anhydrous sodium sulphate and rotaevaporated. The crude product was purified by column chromatography by using the solvent system chloroform – acetone (8:1) to give yellow solid of 10-(3'-N-Chloroprophyl)-3-Chloro-4-Flouro acridone (2) (yield 38%, mp 108 ^{0}C). IR Spectrum (cm^{-1}) 2829 (Ar,C-H), 1687 (C=0), 1085 (C-F), and 758 (C-Cl). ^{1}HNMR ($CDCl_3$- d_6)δ: 7.2-8.0(m, 6H, Ar-H), 1.25-1.9(m, 6H, Hk, Hl and Hm).

Synthesis of N^{10}Chloropropyl- 3-Chloro-4-Fluoro acridone derivatives (Piperidine/N-Methyl /Morpholine/Pyridine/Diethylamine/Diethanolamine)

One gram (0.0034 mmole)of 10-(3-N-chloroprophyl)-3-Chloro-4-Fluoro acridone was dissolved in 30ml of anhydrous acetonitrile and 1.13g potassium iodide and 2.18g of potassium carbonate were added and refluxed for 30 minutes. Then added 1.10g (0.34mmole, 1.22ml) of various secondary amines into it slowly and refluxed for 15 hours until a substantial amount of the product was formed as evidenced by TLC. The contents were cooled, diluted with water and extracted with chloroform; the chloroform layer was washed with water thrice, dried over anhydrous sodium sulphate and evaporated to give an oily product. The oily residue was purified by column chromatography using the solvent system chloroform- acetone (8:1) to give a light yellow oil of 10-[3-(N- Respective-propyl)-3–Chloro-4-Flouro] acridone derivatives. An acetone solution of the free base was treated with ethereal hydrochloride to give the hydrochloride salt that was dried over high vacuum to get pure solids of various yields and melting points. IR Spectrum (cm^{-1}) 3435 (OH), 1626 (C=O), 1047 (C-F), and 812 (C-Cl). ^{1}HNMR ($CDCl_3$- d_6)δ: 7.2-8.4(m, 6H, Ar-H), 2.9-3.7(m, 6H, Ha, Hb, and Hm) and 1.70-1.8(m, 6H, Hc, Hd and Hc) and 1.2-1.4(m, 4H, Hk and Hl).

The scheme for the reaction is given in the Scheme 1.



R=Substituted Secondry Amines

10-[3'(N-Substituted butyl)-3-Chloro-4-Fluoro]acridone

Scheme 1

Anticancer activity

Lung Cancer (HepG2) cell line

Lung cancer cell line (HepG2) cell line was procured from Sigma Aldrich Chemicals Pvt Ltd, Bangalore and maintained in Minimum Essential Medium Eagle Medium (EMEM) with 2 mM Glutamine, 1% Non-essential amino acid and 10% Foetal Bovine Serum (FBS), and antibiotic mixture (Penicillin, streptomycin and ampicillin 100 units/ mL) under defined conditions of temperature at 37^{o}C, 95% humidity and 5% CO_2.

Cytotoxicity Assay

The cytotoxic activity of the different newly synthesized compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay.[14] Sorafanib is taken as the standard control. Each chemical was dissolved in phosphate buffer saline (PBS) buffer, sterilized by membrane filtration, and then serially diluted with culture medium to different concentrations. Lung cancer cell line (HepG2) was trypsinised and the cells were counted using haemocytometer following standard procedure. 100µl of the lung cancer cell line at 1 X 10^4 cells/mL was added to poly 96 well plate and incubated at 37^{o}C in a humidified 5% CO_2 incubator. After 24 hours of incubation, the old medium was replaced with fresh medium and 50 µL of the test compounds was added and incubated for 48 hours at 37^{o}C in a humidified 5% CO_2

incubator. 30µl of 0.5% w/v MTT was added and incubated at room temperature for 4 hours. After incubation, 50 µL of acid-isopropanol was added to dissolve the formazan formed and incubated at room temperature for 30 minutes. Then absorbance was taken at 554nm using Bio-Rad micro-titer plate reader. The IC_{50} values were derived using ELISA software (Gen 5.0 Secure). Later, morphology of the cells was observed by phase contrast microscopy using Nikon Eclipse TS100.

Antioxidant assay

DPPH assay

The antioxidant assay was performed using the free radical scavenging activity of (2, 2-diphenyl-1-picryl-hydrazyl-hydrate). About 1 mg of the synthesised compound was dissolved in 1ml of methanol. About 10 mL of 0.1 mM of DPPH was prepared in methanol and stored in cool dark condition until use. Accurately, 1 mL of DPPH was added to different concentration of the synthesised compound. The mixture of DPPH and synthesised compound was incubated at room temperature for 30 minutes in dark, and then the absorbance was measured at 517 nm in UV spectrophotometer. Ascorbic acid was used as a reference and DPPH without the synthesised compound served as negative control. The IC_{50} value of the sample was calculated based on the absorbance. The percentage of inhibition was calculated using the formula,

DPPH scavenging activity (%) = $[1- (A_{sample}/A_{control})] \times 100$

Where, A_{sample} and $A_{control}$ are the absorbace of the sample and the control respectively.

H_2O_2 scavanging activity

30mg of the synthesized compounds and the standard were dissolved separately in 30 ml DMSO to make a stock solution of 1 mg/ml. From the above solutions further dilutions were made to get different concentrations. Taken 1 ml of each dilution of synthesized compounds and standard drug and then added 2 ml H_2O_2 in PBS. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer saline without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the test compounds and standard drug was calculated as follow:

% Scavenged $[H_2O_2] = [(A_0-A_t)/ A0] \times 100$

Where A_0 was the absorbance of the control and At was the absorbance in the presence of the sample and standard. Calculated the IC_{50} values by the graph were plotted between % scavenging on Y axis and concentration on X axis.

Statistical Analysis

All the datas are expressed as Mean ± SEM of the treatments and are analysed by oneway analysis of variance. Dunnett multiple comparison post test were performed with significant value set at $p<0.05$.

RESULTS AND DISCUSSION

The N^{10} substituted acridones compounds were synthesized by Ulmann's condensation method and the reactions were monitored by TLC. The Physiochemical properties like melting point and solubility were determined for all the intermediate and final products. The compounds were further characterized by IR, and ^1H NMR. All the titled compounds were evaluated for *in-vitro* cytotoxic activity, antioxidant activity.

Cytotoxic studies

The cytotoxic studies were performed in HepG2 cell lines. The results for cytotoxic studies are shown in table 1. It Indicates the IC_{50} value of synthesized acridone derivatives by *in- vitro* cytotoxic activity. The synthesized compounds were screened for *in-vitro* cytotoxic activity by MTT assay (Microculture tetrazolium) using HepG2 cell line. Sorafenib was used as a strandard. Data was analysed and the IC_{50} values were derived using ELISA software (Gen 5.0 Secure). According to IC_{50} values all the compound showed significant cytotoxic activity at different dose.

Table 1: IC_{50} value of synthesized acridone derivatives by *in- vitro* cytotoxic activity.

SL. No.	Compound and Mol. Formula	IC_{50} (mg/L)
Control	-	-
1.	$C_{22}H_{26}N_2O.HCl$	0.021 ± 0.005**
2.	$C_{22}H_{27}N_3O.HCl$	0.015 ±0.0061 **
3.	$C_{21}H_{24}N_2O_2.HCl$	0.081 ± 0.0012**
4.	$C_{21}H_{24}N_2O.HCl$	0.078 ±0.0042 **
5.	$C_{21}H_{26}N_2O.HCl$	0.091 ± 0.0062**
6.	$C_{21}H_{26}N_2O_3$	0.097 ± 0.0072**
Standard	Sorafenib ($C_{21}H_{16}ClF_3N_4O_3$)	2.7 ± 0.010

Data was analysed and the IC_{50} values were derived using ELISA software (Gen 5.0 Secure). $p<0.05$ was considered to be significant.

The observations suggested that the compounds synthesized has a significantly less dose as IC_{50} ($p<0.01$) as compared to the standard sorafenib.

The morphological studies (given in Figure 1) by phase contrast microscopy further confirms that the compound treated with the synthesized compounds are slow growing, round shaped, indistinct and detached from the bottom of the plate. These results suggested that after

24 h of the treatment with the synthesized compound significantly reduced the growth of HepG2 cells. The cytotoxic activity of the compounds as suggested by Fukui et al may be due to the planar structure of the acridones that binds directly to the DNA.[9]

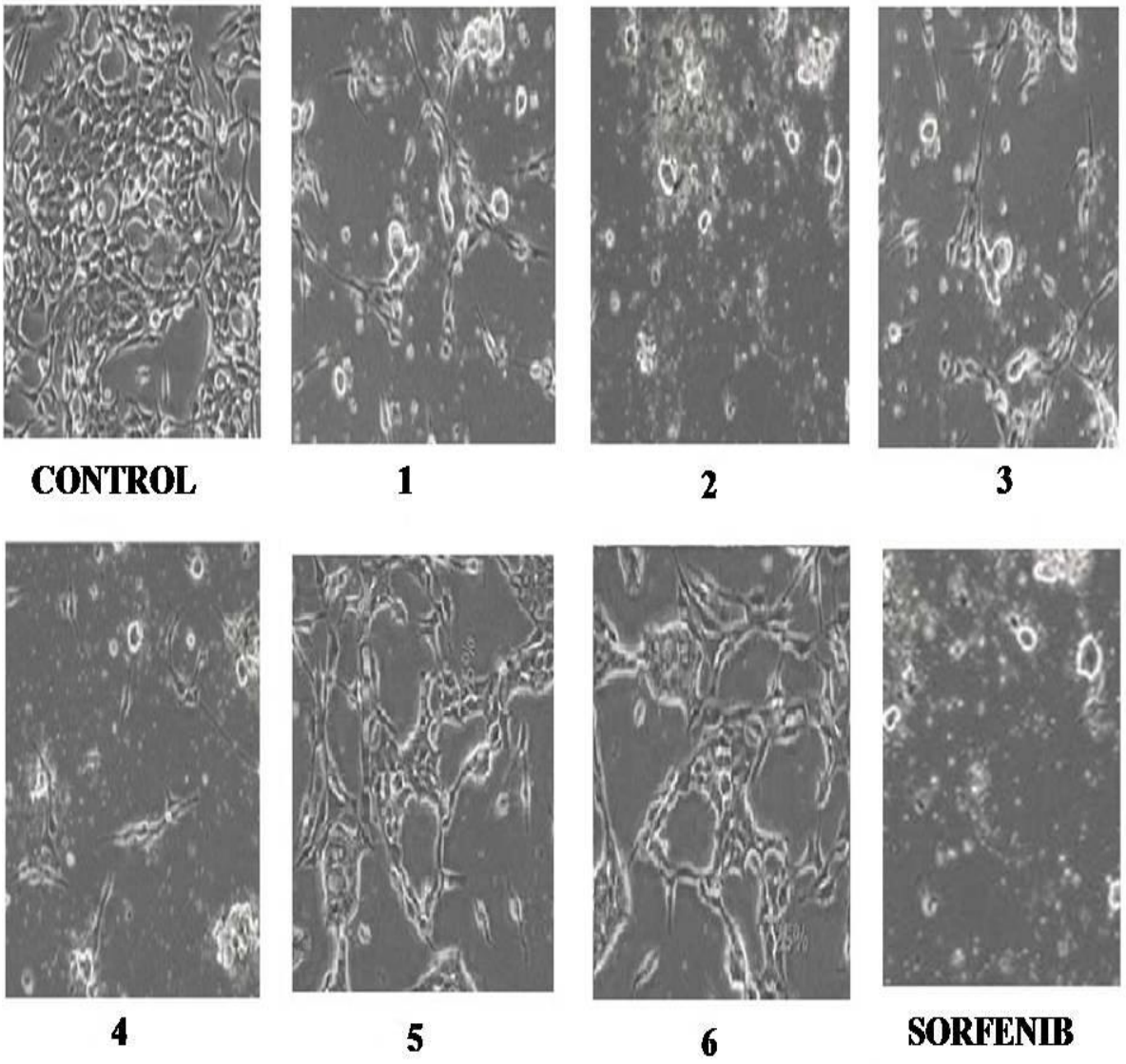


Fig. 1: Phase contrast microscopy of HepG2 cells after treatment of synthesized acridone derivatives.

Antioxidant activity

Further to see the effect of the synthesized compound on antioxidant activity DPPH and H_2O_2 scavenging activity was performed using Ascorbic acid as a standard drug. The graphs were plotted between concentration and % inhibition to calculated the IC_{50} value. Table 2 shows the effect of synthesized acridone derivative on *in-vitro* antioxidant activity by DPPH and H_2O_2 scavenging method.

The result of antioxidant activity suggested that IC_{50} of all the compounds are highly significant as compared to the standard Ascorbic acid ($p<0.01$) inferring that the antioxidant property of the synthesized compounds lies on significantly high dose. Another observation from its antioxidant property is that the compounds having substitutes of heterocylic ring system on 10-[3'- (N-substituted butyl)-3-chloro-4-fluoro]-acridones (ie compound 1, 2, 3 and 4) has shown better antioxidant activity whereas compounds having substitutes of long alkyl chain (compound 5 and 6) has no antioxidant property. This may be due to the ability of heterocyclic moiety to quench the free radical generated due to the electron donation or it acts as a reducing agent in the ring making it comparatively better than long chain alkyl groups which are electron deficient.

Table 2: DPPH and H_2O_2 scavanging activity of synthetic derivatives.

	molecular formula	DPPH free radical scavanging activity		H_2O_2 scavanging activity	
		% Inhibition	IC_{50} (mg/ml)	% Inhibition	IC_{50} (mg/ml)
1	$C_{22}H_{26}N_2O.HCl$	0.53 - 78.3	124 ± 1.6***	0.53 - 78.3	124 ± 1.6***
2	$C_{22}H_{27}N_3O.HCl$	0.46 - 64.7	365 ± 0.67***	0.46 - 64.7	365 ± 0.67***
3	$C_{21}H_{24}N_2O_2.HCl$	2.2 – 92.7	155 ± 0.86***	2.2 – 92.7	155 ± 0.86***
4	$C_{21}H_{24}N_2O.HCl$	0.66- 47.3	345 ± 1.86***	0.66- 47.3	345 ± 1.86***
5	$C_{21}H_{26}N_2O.HCl$	0.66 – 47.3	> 440	0.66 – 47.3	> 440
6	$C_{21}H_{26}N_2O_3$	1.78 – 49.1	>470	1.78 – 49.1	>470
Standard	Ascorbic Acid	45 – 98	13.33 ± 0.88	45 – 98	13.33 ± 0.88

The results are expressed as mean ±SEM. $p<0.05$ is considered to be significant.

CONCLUSION

The investigation suggested that the 10-[3'- (N-substituted butyl)-3-chloro-4-fluoro]-acridones has a potent cytotoxic property in HepG2 cells that can be further explored for its use in HCC. However, the synthesized compounds exhibited its antioxidant property at a very high dose.

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