

CHEMISTRY & BIOLOGY INTERFACE

An official Journal of ISCB, Journal homepage; www.cbijournal.com

In Vitro Antimicrobial, Antiproliferative, and Antioxidant Activities of Bis Pyrrolizidine Fused Dispiro Oxindolo Curcuminoids

Yogesh P. Bharitkar^{1†}, Suhana Datta^{2†}, Saurov Sett², Neeraja Marathe², Preeti Khan², Abhijit Hazra^{1*}, Meenakshi Singh¹, Ashutosh Sahoo¹, Shekhar Ghosh³, Susanta Mondal⁴, Arup K. Mitra², Ravichandiran V.¹, Nirup B. Mondal^{3*}

¹National Institute of Pharmaceutical Education and Research (NIPER), 4 Raja S. C. Mullick Road, Jadavpur, Kolkata - 700 032, India.

²P.G. Dept of Microbiology, St. Xavier's College, Kolkata-700017, India.

³Department of Organic and Medicinal Chemistry, Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, 4 Raja S. C. Mullick Road, Jadavpur, Kolkata - 700 032, India.

⁴TCG Life Sciences Ltd, BIPL, Block-EP & GP, Sec-V, Salt Lake, Kolkata-700091, India.

^{†*}Corresponding author(s) E.mail: nirup@iicb.res.in/apuhazra@gmail.com;

Telephone: +91 33 2473 3491/3493/6793; Fax: (033) 2473 5197, 2472 3967

Received 13 December 2016; Accepted 20 February 2017

Abstract: Curcumin has been transformed to racemic/meso curcuminoids via an azomethine ylide cycloaddition reaction using isatin/acenaphthoquinone and proline. The resulting analogues were subjected to screening of antimicrobial efficacy against some opportunistic pathogenic bacteria (*B. subtilis*, *E. coli*, *P. aeruginosa*, *S. aureus*) and the potent ones (**4Dd**, **4Ed**, **4Fb** and **4Fd**) were assessed for growth curve assay and biomolecular damages. Cytotoxicity studies against six cell lines (Caco-2, MDCK-2, CHO-K1, HEK-293, Hela and HepG-2) revealed that IC₅₀ values of some derivatives (**3Ab**, **4Ba**, **4Bb**, **4Da**, **4Db** and **4Fc**) were markedly lower than those of curcumin in all the six cell lines, the products being most potent against Hek-293, Hela, and Hep-G2. DPPH radical scavenging activity showed significant antioxidant potential in these analogues compared to curcumin.

Keywords: Dispirocurcuminoids; Antimicrobial; Anticancer; DPPH, MTT

Introduction:

Curcumin, derived from the rhizome of *Curcuma longa* (common name turmeric), is one of the best gifts nature has bestowed upon mankind. From time immemorial it is being used as a colouring

spice in almost all the kitchens of the Indian sub-continent and also finding applications in traditional medicines for many remedies.^{1,2} The rhizomes of *C. longa* have been used by the traditional healers as anti-inflammatory³ and insect repellent, and for wound healing.⁴

There are several reports on the broad-spectrum antimicrobial activity of curcumin, namely, antibacterial, antiviral, and antifungal, along with its antimalarial, anti-oxidant, anti-cancer, and anti-HIV activities.⁵⁻⁹ However, poor water solubility, relatively low bioavailability, unsatisfactory pharmacokinetics and colour stain^{10,16} of curcumin have restricted its utility as a drug candidate. In order to overcome these difficulties, a new concept of 'Super Curcumins' has been evolved and such products may be classified under two broad categories, viz. synthetic analogues and formulations,¹⁷⁻¹⁹ which do not have these drawbacks while the efficacy is equal to or better than that of curcumin. We decided to pursue candidates belonging to the first category, *i.e.* synthetic analogues or derivatives. Literature survey revealed that several research groups have synthesized compounds such as pyrazole/isoxazole fused derivatives,^{20, 21} metal conjugates,²²⁻²⁴ and PEGylated/ferrocenylated²⁵⁻²⁷ curcuminoids by using the diketone functionality, phenolic OH group, and the active methylene group, but no attempt appears to have been made to exploit the electron deficient double bonds. We, for the first time, conceptualized reactions at this target, designed reaction protocols, and successfully implemented the transformation of curcumin to racemic/meso curcuminoids via an azomethine ylide cycloaddition reaction using isatins/acenaphthoquinone and proline. At the outset, thirty six (36) curcumin analogues were prepared and characterized with detailed spectral analysis.²⁸ The preparative simplicity of the methodology and structural novelty of the molecules demanded their thorough evaluation for biological and therapeutic activity. In this endeavor, *in vitro* antibacterial, antiproliferative and antioxidant activity of the molecules were investigated. Here we wish to report the antimicrobial activity of the analogues against several opportunistic pathogenic bacteria, antiproliferative activity against several human cancer cell lines and radical scavenging activity

compared with curcumin itself.

Materials and methods

Measurement of Antimicrobial activity: The title compound curcumin and its synthesized derivatives were evaluated for their antibacterial activity against *Staphylococcus aureus* MTCC 3160 and *Bacillus subtilis* MTCC 9041 (as Gram-positive bacteria), as also *E.coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 741 (as Gram-negative bacteria) by agar cup plate method to primarily identify the potent compounds.²⁹ The test compounds were dissolved in DMSO to prepare a stock concentration of 1mg/mL, while the antibiotics were dissolved in sterile distilled water to prepare a similar stock solution. Stock concentrations of each test compound were further diluted within the range of 15.6-500 µg/mL. Selected bacterial culture was inoculated in sterile nutrient broth and was incubated for 24 h at 37 °C. A 100 µL aliquot of bacteria culture (inoculum) was spread on Mueller Hinton Agar (Hi Media). Stock concentrations of the test compounds were added in the specific wells and zones of inhibition (ZOI) were measured after 24 h of incubation. DMSO was used as negative control and antibiotics (Streptomycin, Tetracylin) as positive control.³⁰

Minimum inhibitory concentrations (MIC) of selected compounds with maximum ZOI were measured. Different dilution of selected test compounds were mixed in 5 mL nutrient broth and 0.05 mL of active bacterial culture was added to each tube to make a final inoculum of 5×10^5 CFU/mL. The tubes were incubated aerobically at 37 °C up to 24 h. The lowest concentration of the compound that completely inhibits bacterial growth (no turbidity) in comparison to control was regarded as MIC. DMSO was shown not to affect the growth of the bacteria during the experiments.^{31,32}

Determination of bacterial motility: Bacterial

motility was observed by hanging drop method using phase contrast microscope.³⁰

Growth kinetic study: The rate and extent of bacterial killing effect were studied by time dependent growth kinetics assay. The log phase bacterial suspension (0.05 mL, 5×10^5 CFU/mL) was added into 5 mL NB containing selected compounds at its IC_{50} dose compared to control. Tubes were incubated at 37 °C. Absorbance was taken at different time intervals (0, 2, 4, 6, 8, 10, 12, 24 h). A 24 h time kill curve was constructed for each compound at each interval.

Measurement of bacterial lipid damage: Lipid damage was measured in terms of malonaldehyde (MDA) in the bacterial lysate using the modified method of Beuge and Aust. Briefly, one volume bacterial lysate was mixed thoroughly with 2 volumes of 15% w/v TCA, 0.375% w/v TBA and 0.25N HCl. The mixture was incubated at 100 °C for 30 min in a shaking water bath; it was then cooled and the flocculent precipitate removed by centrifugation (1000g for 10 min, 20-25°C). The absorbences were determined at 535 nm and concentration of thiobarbituric acid reactive substances (TBARS) was calculated using $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ as molar extinction coefficient of MDA.³³

Measurement of bacterial protein damage: Protein carbonyls are first derivatised with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2M HCl. Briefly, 50 µg of protein lysates were incubated with 100 µL DNPH solution for 30 min in dark. Proteins were then precipitated with 100 µL 20% TCA and free DNPH was removed by washing the protein pellet with (1:1) ethanol-ethyl acetate solution. The protein pellet was then dissolved in 350 µL of 2% sodium dodecyl sulphate (SDS) and the absorbance of protein-hydrozone was measured spectrophotometrically at 370 nm. Carbonyl concentration was calculated from the maximum absorption (relative to the reagent blank) using

a value for the extinction coefficient of $22\,000 \text{ M}^{-1}\text{cm}^{-1}$ and expressed as nmoles of carbonyl per mg of protein.³⁴

Antioxidant study

Measurement of radical scavenging activity

Antiradical activity was measured by a decrease in absorbance at 517 nm of DPPH (2,2-Diphenyl-1-Picrylhydrazyl) solution. In this assay DPPH acts as an indicator for “Radical Scavenging Activity” and changes its deep violet colour to colourless or pale yellow in presence of antioxidant.³⁵ Therefore, to determine RSC of the extracts, 1mL of DPPH (0.1mM) solution was mixed with 2 mL of test compound solution in methanol of varying concentration (15.6—500 µg/mL) and kept for 20 min incubation in dark. After 20 min, absorbance was measured at 517 nm. Decrease in the absorbance of the DPPH solution indicates an increase of the DPPH antioxidant activity, and the percentage of Radical Scavenging Activity (% RSC) was calculated as $(A_o - A_s)/A_o \times 100$ [A_o = absorbance of DPPH solution without the sample, A_s = absorbance with the sample].

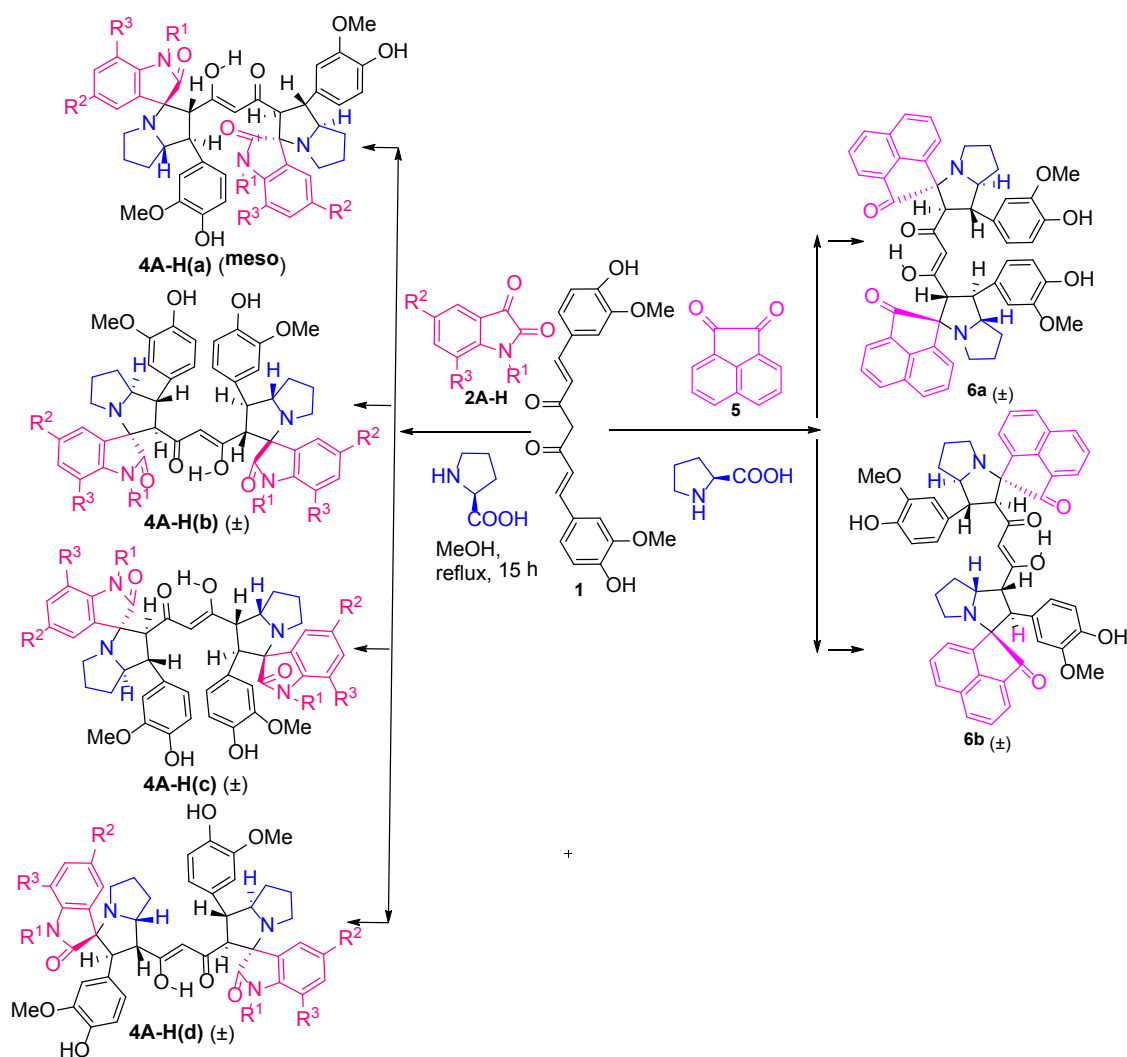
Anticancer study: Determination of cytotoxicity

Cell line maintenance: Cancer cell lines were obtained from ATCC (American Type Culture Collection, USA). For maintenance of cell lines, Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) of Gibco, antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), and amphotericin (0.25 µg/mL) of Hi-Media was employed. The cells were maintained in cell culture flasks in CO₂ incubators at 37 °C with 5% CO₂ in air and 99% humidity. Passaging of cells when confluent was carried out using 0.25% trypsin and 0.02% EDTA (Hi-Media) in phosphate buffered saline (PBS).

MTT assay: Cell viability after incubating the cells with different concentrations of the test substance was determined by methyl thiazolyl tetrazolium (MTT) assay. It is a colorimetric assay based on the ability of live, but not dead cells to reduce MTT (yellow) to a formazan (purple) product. The cells were spread in 96-well plates at 5×10^5 cells/well. After 36 h of seeding, they were incubated with different concentrations (15.6–500 $\mu\text{g/mL}$) of the test substance individually for 24 h. Subsequently, the cells were exposed to MTT at a concentration of 50 $\mu\text{g/well}$ for 2.5 to 3 h at 37°C in a CO_2 incubator. The working solution of MTT was

prepared in Hanks balanced salt solution (HBSS). When visible under the microscope, the formazan crystals were solubilized by treating the cells with DMSO: isopropanol at a ratio of 1:1 for 20 min at 37°C . The plate was read at an absorbance of 570 nm. The relative cell viability in percent was calculated as (Absorbance of treated/Absorbance of control) $\times 100$. Cells without any treatment were used as control. Cell viability of control cells were kept as 100%.

Statistical analysis: All experiments were carried out in triplicate. Data obtained was



Scheme 1. Preparation of bis-pyrrolizidine fused di-spiro oxindolo/acenaphthylene curcuminoids

Table 1. Yields of analogues 4A-H(a-d) from curcumin

Entry	R ¹	R ²	R ³	Product	Yield (%) ^a
1	H	H	H	4Aa	18
	H	H	H	4Ab	23
	H	H	H	4Ac	21
	H	H	H	4Ad	28
				3Aa^b	85
				3Ab^b	5
2	H	I	H	4Ba	19
	H	I	H	4Bb	23
	H	I	H	4Bc	21
	H	I	H	4Bd	27
3	H	F	H	4Ca	17
	H	F	H	4Cb	21
	H	F	H	4Cc	20
	H	F	H	4Cd	28
4	H	Cl	H	4Da	17
	H	Cl	H	4Db	23
	H	Cl	H	4Dc	21
	H	Cl	H	4Dd	28
5	H	Me	H	4Ea	18
	H	Me	H	4Eb	22
	H	Me	H	4Ec	22
	H	Me	H	4Ed	28
6	H	Me	Me	4Fa	18
	H	Me	Me	4Fb	23
	H	Me	Me	4Fc	21
	H	Me	Me	4Fd	27
7	Me	H	H	4Ga	19
	Me	H	H	4Gb	23
	Me	H	H	4Gc	22
	Me	H	H	4Gd	27
8	H	OMe	H	4Ha	16
	H	OMe	H	4Hb	22
	H	OMe	H	4Hc	21
	H	OMe	H	4Hd	26

^aIsolated yield. ^bCompound numbering as reported in Ref 28.

analyzed by one-way analysis of variance, and mean was compared by Duncan's tests. Differences were considered significant at $P < 0.05$.

Results and discussion

Chemistry

As described recently,²⁸ the cycloaddition reaction of curcumin (**1**), isolated from *Curcuma longa*, was accomplished using proline along with isatins (**2A-H**) or acenaphthoquinone (**5**) via in situ generation of azomethine ylides. The reactions produced four stereoisomeric bis-pyrrolizidine fused-di-spiro oxindolo curcuminoid products **4A-H(a-d)** in 14–16 h with yields of 85-90% via the formation of regioisomeric monocycloaddition products (**3Aa**, **3Ab**). In case of acenaphthoquinone (**5**), we obtained the anticipated curcuminoids **6a** and **6b** in around 76% yield (Scheme 1, Table 1). The products were characterized by extensive 1D/2D NMR (HMBC, COSY, NOESY) analysis, HRMS and few single-crystal X-ray crystallographic studies.

Pharmacology

Antimicrobial activity

At the very first step antibacterial activity evaluations of the curcuminoid analogues were carried out in vitro against the Gram-positive bacteria *Staphylococcus aureus* MTCC 3160 and *Bacillus subtilis* MTCC 9041, and Gram-negative bacteria *E. coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 741 by agar cup plate method.²⁹ Stock solutions (1 mg/mL), prepared by dissolving the curcuminoids in DMSO and those for the antibiotics in sterile distilled water, were further diluted to 15.6-500 µg/mL. Selected bacterial culture inoculated in sterile nutrient broth was incubated for 24 h at 37 °C. An aliquot (100 µL) of inoculum

(bacterial culture) was spread on Mueller Hinton Agar (Hi Media), stock concentrations of the test compounds were added in the specific wells, and zones of inhibition (ZOI) were measured after 24 h of incubation. DMSO was used as negative control and antibiotics (Streptomycin, Tetracylin) as positive control.³⁰ The determined ZOI against selected bacteria are shown in Table 1. Maximum values (> 20 mm) were observed for **4Dd**, **4Ed**, **4Fb** and **4Fd**. These were further tested for MIC and the results are given in Table 2. The ranges of MIC for the test pathogens were 15.6 to 250 µg/mL for *E. coli*, 62.5-250 µg/mL for *B. subtilis*, 15.6 to 125 µg/mL for *P. aeruginosa*, and 15.6-62.5 µg/mL for *S. aureus*. In this study, **4Fd** displayed the highest activity followed by **4Ed**, while **4Dd** showed the least activity. Thus **4Ed**, **4Fb** and **4Fd** were further selected for growth curve assay.

Table 2: IC₅₀ values of test compounds

Organism	IC ₅₀ value (µg/mL)			
	4Dd	4Ed	4Fb	4Fd
<i>E. coli</i>	-----	15.6	250	15.6
<i>B. subtilis</i>	250	62.5	500	62.5
<i>P. aeruginosa</i>	15.6	15.6	-----	125
<i>S. aureus</i>	-----	31.25	62.5	15.6

Effect on bacterial motility

Effect on bacterial motility was determined by the hanging drop method using phase contrast microscope.³⁰ At the IC₅₀ dose of selected compounds, the reduction of motility was highest for **4Ed** and **4Fd** considering all tested organism (Figure 1).

Table 1: Antibacterial activity of the test compounds and antibiotics against bacteria

Compd No	ZOI in mm for				DPPH (RSC in $\mu\text{g/mL}$)	Inference
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>		
4Aa	18.5 $\pm$ 0.5	-	-	-	*	Low activity
4Ab	22.5 $\pm$ 0.3	19.5 $\pm$ 0.3	-	-	x	Highly sensitive to Gram -ve
4Ac	16 $\pm$ 0.5	-	-	-	x	Low activity
4Ad	-	-	-	-	x	No activity
3Aa	-	-	-	-	525 $\pm$ 10.5	Low antioxidant
3Ab	-	-	-	-	205 $\pm$ 15.5	High antioxidant
4Ba	18.5 $\pm$ 0.3	-	15.5 $\pm$ 0.3	-	258 $\pm$ 20.5	Moderate antibacterial, high antioxidant
4Bb	15 $\pm$ 0.5	18 $\pm$ 0.2	-	-	x	Low antibacterial
4Bc	-	-	-	-	x	No activity
4Bd	-	-	-	-	x	No activity
4Ca	14.5 $\pm$ 0.5	15 $\pm$ 0.5	-	-	x	Low antibacterial
4Cb	17.5 $\pm$ 0.3	15.6 $\pm$ 0.5	17.5 $\pm$ 0.5	-	x	Low antibacterial
4Cc	21.5 $\pm$ 0.5	18.5 $\pm$ 0.3	18 $\pm$ 0.3	-	x	High sensitivity to gram-ve
4Cd	-	-	18.5 $\pm$ 0.5	-	795 $\pm$ 32.5	Low antibacterial, moderate antioxidant
4Da	17.5 $\pm$ 0.5	-	16.5 $\pm$ 0.5	16.5 $\pm$ 0.5	325 $\pm$ 19.8	Moderate antibacterial and antioxidant
4Db	19.5 $\pm$ 0.3	-	19 $\pm$ 0.2	18.5 $\pm$ 0.5	285.5 $\pm$ 12.6	Moderate antibacterial and antioxidant
4Dc	16.5 $\pm$ 0.5	16 $\pm$ 0.3	-	15.5 $\pm$ 0.4	175.8 $\pm$ 9.8	Moderate antibacterial and high antioxidant
4Dd	22.5 $\pm$ 0.5	18.5 $\pm$ 0.3	21 $\pm$ 0.5	-	155.7 $\pm$ 16.2	Moderate to high antibacterial, high antioxidant
4Ea	22 $\pm$ 0.3	21.5 $\pm$ 0.5	21 $\pm$ 0.5	-	x	Moderate antibacterial
4Eb	-	-	-	+	325 $\pm$ 21.5	Moderate antioxidant
4Ec	-	19.5 $\pm$ 0.2	-	-	102.5 $\pm$ 10.4	High antioxidant
4Ed	21.5 $\pm$ 0.3	25.5 $\pm$ 0.5	19.5 $\pm$ 0.5	20.2 $\pm$ 0.3	96.2 $\pm$ 5.5	Moderate antibacterial, high antioxidant
4Fa	19.5 $\pm$ 0.6	18 $\pm$ 0.5	-	-	x	Low antibacterial
4Fb	20.5 $\pm$ 0.4	23.5 $\pm$ 0.5	19 $\pm$ 0.6	18.6 $\pm$ 0.3	95.5 $\pm$ 5.2	Moderate to high antibacterial, high antioxidant
4Fc	20.5 $\pm$ 0.2	19.6 $\pm$ 0.3	-	21.5 $\pm$ 0.4	x	Low antibacterial
4Fd	24.5 $\pm$ 0.4	20.5 $\pm$ 0.5	18.6 $\pm$ 0.5	24.5 $\pm$ 0.3	76.2 $\pm$ 7.5	High antibacterial, high antioxidant
4Ga	-	-	-	-	X	No activity
4Gb	14.5 $\pm$ 0.5	-	-	-	X	Moderate sensitive to gram-ve
4Gc	12.5 $\pm$ 0.4	-	-	-	X	Moderate sensitive to gram-ve
4Gd	-	-	-	-	x	No activity
4Ha	13.2 $\pm$ 0.5	-	-	-	x	No activity
4Hb	-	-	-	-	x	No activity
4Hc	-	-	-	-	x	No activity
4Hd	-	-	-	-	x	No activity
6a	-	-	-	-	x	No activity
6b	15.5 $\pm$ 0.6	-	-	-	200.1 $\pm$ 18.6	Low antibacterial, high antioxidant
1	16.5 $\pm$ 0.2	16.5 $\pm$ 0.3	-	-	50.2 $\pm$ 4.5	Moderate antibacterial, very high antioxidant
Streptomycin	Not tested	24.5 $\pm$ 0.3	-	25.2 $\pm$ 0.4	NA	
Tetracyclin	-	-	27.2 $\pm$ 0.4	27 $\pm$ 0.5	NA	

RSC: Radical Scavenging Activity

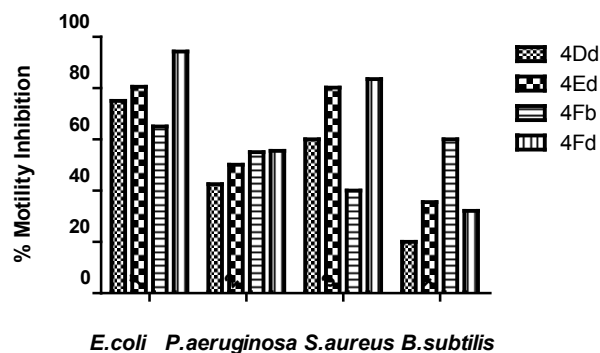


Figure 1: Bacterial motility assay

Growth curve study

Growth kinetics study was conducted to determine the action of selected derivatives against the test organisms. The results revealed that the growths of these organisms decreased with increasing concentration of compounds, reaching 50% inhibition after 12 h time interval (Figure 2).

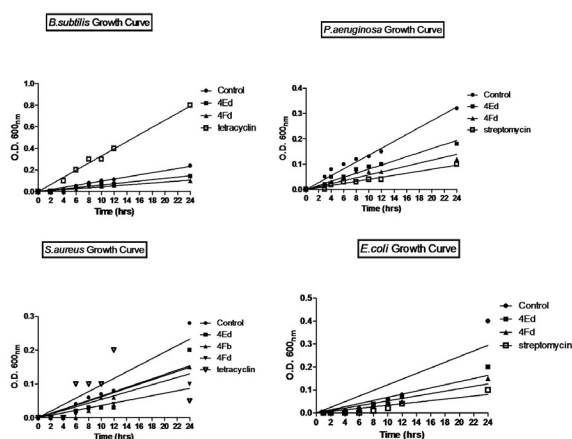


Figure 2: Growth kinetics study

Extent of membrane lipid damage

The selected molecules were then subjected for lipid peroxidation studies. Lipid peroxidation occurs following the attack of polyunsaturated fatty acids of the membrane by several reactive oxygen species (ROS) and initiates a self-propagating chain reaction which culminates

in the destruction of membrane lipids and loss of cell viability. The current study revealed an increased amount ($p < 0.001$) of lipid peroxidation (as measured in terms of MDA) for all tested bacteria when treated with IC_{50} concentration of **4Ed**. The effect was also significantly high ($p < 0.05$) for all tested bacteria when treated with IC_{50} of **4Dd** and **4Fd** (Figure 3).

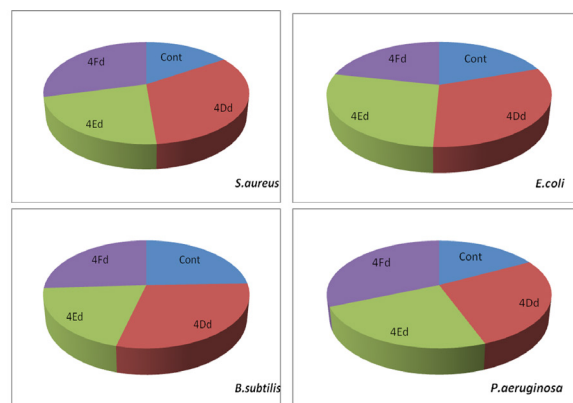


Figure 3: Lipid peroxidation study of selected curcumin derivatives in bacterial isolates

Extent of protein damage

It has been established that carbonyl groups are introduced into proteins after ROS react with the amino group of lysine residues. Consequently the degree of carbonylation is an effective indicator of the extent of ROS induced oxidative modification of proteins. A substantial amount of protein carbonylation was observed in bacterial lysates when treated with IC_{50} dose of **4Ed**. It was found to be 1.5-2.0 fold higher ($p < 0.001$) when compared with the control. The increase was 1.5 fold ($p < 0.05$) higher when treated with IC_{50} doses of **4Fb** and **4Fd** (Figure 4).



Figure 4: Protein carbonylation study of curcumin derivatives in bacterial isolates

Radical scavenging assay

The DPPH radical scavenging activities are shown in Table 1. The concentration for 50% inhibition (IC_{50}) was the parameter used to compare the activities, a lower IC_{50} value implying better radical scavenging activity. The findings reveal that significant DPPH radical scavenging activity compared to control (IC_{50} value $<150 \mu\text{g/mL}$) was present in **4Ed**, **4Fb**, and **4Fd** (Figure 5).

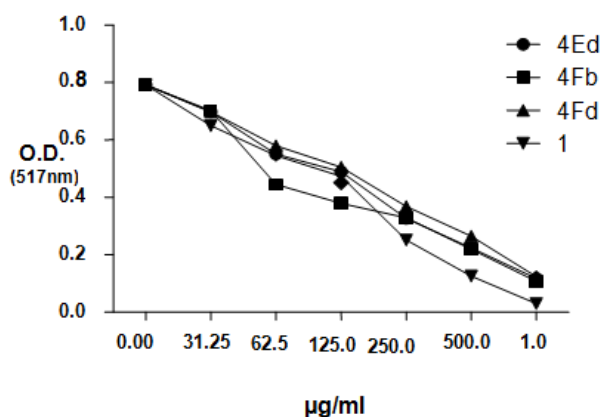


Figure 5: Radical scavenging assay of curcumin derivatives

Anticancer study

All the curcuminoid analogues along with curcumin were then tested in six human cancer cell lines (Caco-2, MDCK-2, CHO-K1, HEK-293, Hela and HepG-2) to assess their

cytotoxicity potential. As seen in Table 3, IC_{50} values of some analogues like **4Fc**, **4Fd**, **3Ab**, **4Ba**, **4Bb**, **4Da**, and **4Db** were markedly lower than those of curcumin in all the six cancer cell lines tested. However, the degree of cytotoxicity varied among the cell lines, the compounds being most potent on Hek-293, Hela, and Hep-G2. Effects of these potent curcuminoid derivatives were dose and time dependent within the concentration range of 0-25 μM .

As a part of our ongoing research program on bioprospection of natural products,³⁶⁻⁴¹ the hybrid curcuminoids were screened in this study for potent therapeutic activity. The results reveal that the majority of the derivatives show varying degree of inhibitory activity against the tested organisms, though some were inactive and some showed more potency towards Gram +ve bacteria. The antibacterial activity of curcumin has been reported earlier by several groups of researchers. However, this study revealed that compounds **4Dd**, **4Ed**, **4Fb** and **4Fd** possess higher activity ($ZOI >20 \text{ mm}$) against the tested organism (Table 1). The results are comparable to those of the reference antibiotics streptomycin and tetracycline. The selected compounds were subjected to MIC testing also. The findings summarized in Table 2 show that the growths of these organisms decreased with increasing concentrations of compounds, reaching 50% inhibition after 12 h. At the IC_{50} doses of selected compounds, the reduction of motility was highest for **4Ed** and **4Fd** against all tested organism compared to **4Dd** and **4Fb** (Figure 2).

Physiological amounts of reactive oxygen species (ROS) are a mandatory cellular requirement to affect signalling pathways, regulating a variety of cellular activities including cytokine secretion, growth, differentiation and host defence against invading pathogens. However, ROS can be a double edged sword as it concomitantly has the potential to induce

Table 3: Anticancer study of curcumin derivatives on tumour cell line

SL	Compound ID	IC50 (μ M)					
		Caco-2	MDCK-II	CHO-K1	HEK-293	Hela	HepG2
1	1	14.9$\pm$0.0	16.0$\pm$0.4	15.5$\pm$0.0	13.6$\pm$0.0	13.6$\pm$0.0	14.1$\pm$0.3
2	4Aa	NA	NA	NA	33.3 $\pm$ 0.6	26.5 $\pm$ 2.9	31.9 $\pm$ 1.3
3	4Ab	NA	NA	NA	62.5 $\pm$ 2.8	33.2 $\pm$ 0.3	NA
4	4Ac	NA	NA	NA	NA	NA	NA
5	4Ad	NA	NA	42.5 $\pm$ 5.7	NA	32.0 $\pm$ 3.5	32.3 $\pm$ 0.7
6	3Aa	33.4 $\pm$ 6.3	11.8 $\pm$ 0.0	15.8 $\pm$ 0.6	17.9 $\pm$ 2.0	20.5 $\pm$ 0.1	20.8 $\pm$ 0.0
7	3Ab	20.9$\pm$1.7	8.8$\pm$0.0	10.9$\pm$0.0	6.9$\pm$0.2	6.2$\pm$0.4	12.5$\pm$0.2
8	4Ba	NA	21.3 $\pm$ 10.8	43.0 $\pm$ 8.6	7.6$\pm$0.5	5.9$\pm$0.1	11.0$\pm$0.0
9	4Bb	NA	13.7 $\pm$ 1.6	14.7 $\pm$ 3.6	6.5$\pm$0.1	6.5$\pm$0.1	9.8$\pm$1.1
10	4Bc	NA	NA	NA	13.2 $\pm$ 2.1	8.9$\pm$0.4	12.2$\pm$0.0
11	4Bd	NA	NA	32.4 $\pm$ 11.4	12.0 $\pm$ 2.2	10.4 $\pm$ 0.7	13.3 $\pm$ 0.0
12	4Ca	NA	35.2 $\pm$ 2.2	NA	18.5 $\pm$ 0.6	14.3 $\pm$ 0.8	18.1 $\pm$ 2.4
13	4Cb	NA	33.3 $\pm$ 0.6	NA	29.3 $\pm$ 4.6	16.5 $\pm$ 0.1	27.7 $\pm$ 3.3
14	4Cc	NA	NA	NA	37.6 $\pm$ 0.3	30.1 $\pm$ 0.6	46.9 $\pm$ 9.8
15	4Cd	NA	24.7 $\pm$ 7.3	44.6 $\pm$ 12.5	13.3 $\pm$ 0.2	14.8 $\pm$ 0.3	28.2 $\pm$ 0.1
16	4Da	NA	16.1 $\pm$ 1.2	31.1 $\pm$ 3.1	9.9$\pm$1.2	7.4$\pm$0.1	11.7$\pm$1.3
17	4Db	NA	15.9 $\pm$ 0.1	16.1 $\pm$ 1.7	9.2$\pm$0.7	7.5$\pm$0.0	8.8$\pm$1.1
18	4Dc	NA	33.9 $\pm$ 1.5	30.9 $\pm$ 3.1	17.8 $\pm$ 0.4	13.5 $\pm$ 0.0	21.8 $\pm$ 6.0
19	4Dd	NA	32.5 $\pm$ 1.3	28.2 $\pm$ 1.2	15.9 $\pm$ 0.4	13.1 $\pm$ 0.2	16.7 $\pm$ 0.6
20	4Ea	58.3 $\pm$ 5.1	27.0 $\pm$ 1.9	51.7 $\pm$ 7.2	22.4 $\pm$ 5.2	15.7 $\pm$ 0.1	16.4 $\pm$ 0.4
21	4Eb	59.7 $\pm$ 3.6	17.2 $\pm$ 0.5	24.2 $\pm$ 3.8	15.8 $\pm$ 1.5	14.1 $\pm$ 0.1	14.6 $\pm$ 0.3
22	4Ec	NA	34.4 $\pm$ 4.0	59.5 $\pm$ 0.7	30.4 $\pm$ 0.2	21.7 $\pm$ 1.1	29.3 $\pm$ 0.2
23	4Ed	NA	36.2 $\pm$ 0.7	29.8 $\pm$ 0.2	20.8 $\pm$ 4.1	14.4 $\pm$ 0.0	23.1 $\pm$ 5.5
24	4Fa	NA	15.2 $\pm$ 2.8	28.9 $\pm$ 0.2	14.8 $\pm$ 0.3	14.4 $\pm$ 0.0	14.8 $\pm$ 0.2
25	4Fb	NA	14.4 $\pm$ 2.3	17.2 $\pm$ 0.3	13.9 $\pm$ 0.0	13.6 $\pm$ 0.1	13.1 $\pm$ 1.0
26	4Fc	25.0$\pm$5.2	8.2$\pm$0.5	8.6$\pm$0.3	7.5$\pm$0.0	6.1$\pm$0.0	6.1$\pm$0.0
27	4Fd	NA	14.4 $\pm$ 1.8	16.0 $\pm$ 0.8	10.3$\pm$1.7	7.8$\pm$0.0	11.5$\pm$2.6
28	4Ga	NA	14.1 $\pm$ 2.8	40.9 $\pm$ 1.3	34.2 $\pm$ 1.0	34.9 $\pm$ 1.2	53.9 $\pm$ 0.9
29	4Gb	NA	NA	19.5 $\pm$ 1.4	46.6 $\pm$ 0.3	21.3 $\pm$ 5.4	29.6 $\pm$ 1.4
30	4Gc	NA	NA	27.9 $\pm$ 0.1	NA	24.9 $\pm$ 5.6	31.9 $\pm$ 3.0
31	4Gd	NA	NA	31.9 $\pm$ 0.6	51.9 $\pm$ 10.6	26.6 $\pm$ 0.3	36.7 $\pm$ 6.4
32	4Ha	NA	NA	NA	NA	57.6 $\pm$ 0.3	NA
33	4Hb	NA	NA	NA	NA	31.6 $\pm$ 0.8	55.4 $\pm$ 5.6
34	4Hc	NA	NA	NA	NA	NA	NA
35	4Hd	NA	NA	NA	NA	55.4 $\pm$ 1.8	NA
36	6a	NA	NA	NA	28.0 $\pm$ 0.9	56.9 $\pm$ 0.4	NA
37	6b	NA	NA	NA	NA	NA	NA

NA= Not Active (IC50 $\geq$ 60)

significant biological damage when it counters the cells robust antioxidant system. Current research is now directed towards finding naturally occurring antioxidants which could reduce or minimize the deleterious effect caused by ROS. The results of our investigation reveal that some hybridized curcuminoids show significant antioxidant capacity as determined by DPPH assay.

From the structural features of these spiro-curcuminoids retaining the functional groups of curcumin, inclusion of the bis-pyrrolizidine fused-di-spiro oxindole moiety appears to have enhanced the antibacterial activity⁴²⁻⁴⁹ and antiproliferative potential⁵⁰⁻⁵⁴ compared to curcumin.

As a whole the bioactivity may be due to the combined effect of the curcuminoid skeleton (its existing phenolic hydroxyl associated with generation of radical displaying electron-donating properties, the ortho-methoxy group facilitating the formation of intramolecular hydrogen bond enhancing the stability of the radical), and the bis-pyrrolizidino dispiro-oxindole/acenaphthoquinone ring along with the substituents present.¹⁷

Substituents in aromatic ring also play a major role in bioactivity. Presence of halogen or alkyl group is quite beneficial for bioactivities due to the change in chemical as well as pharmacological properties. But the effect also depends on the type of halogen/alkyl group present. In this study, chlorine and methyl/dimethyl substituted derivatives (**4Dd**, **4Ed**, **4Fb**, **4Fc**, **4Fd**, **3Ab**, **4Ba**, **4Bb**, **4Da** and **4Db**) showed distinctly better antibacterial/ cytotoxic activity than iodine or fluorine substituted ones. Chlorine substituted products benefit from the fact that the halogen possesses both electronegative (-I) and resonance (+R) effect and can occupy hydrophobic pockets of biological target to form a stable and potent complex at specific binding

sites.⁵⁵⁻⁵⁹ Compounds with methyl or dimethyl substitution (**4Ed**, **4Fb**, **4Fc** and **4Fd**) also show high antibacterial and cytotoxic activity. Their strong antioxidant property may be ascribed to the high oxidation potential of methyl groups compared to other substituent's, which may also account for their cytotoxic effect.

Conclusion

In the present study, as a part of our ongoing research on bioprospection of natural products, we have screened curcumin and its 36 hybridized derivatives against opportunistic bacterial pathogens, in human cancer cell lines, and by DPPH assay to evaluate their antibacterial, anticancer and antioxidant activity. The results are promising and the study will have an impact on the prohibitive cost of conventional drugs coupled with the need for long term therapy that results in a high frequency of side effects making it imperative for better and safer drugs to be made available. Further biological studies with more diversified analogous curcuminoids, which are in progress, are needed to fully develop strategies for improving the bioavailability of the super curcumins.

Acknowledgements:

The authors express their gratitude to the Director, IICB and to the Principal, St. Xavier's College for laboratory facilities. Y.P.B. and A.H. are recipients of National Post-Doctoral fellowship (PDF/2016/000088) and Young Scientist Grant (YSS/2015/001141 from SERB, DST, Govt. of India. N.B.M. acknowledges Emeritus Scientist grant [21(0197)/12/EMR-II] from CSIR. Our thanks are due to Dr. B. Achari (Ex-emeritus Scientist, CSIR) for helpful suggestions.

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