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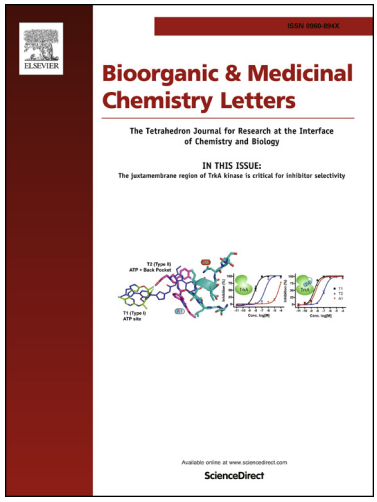

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Synthesis of extended conjugated indolyl chalconesas potent anti-breast cancer, anti-inflammatory and antioxidant agents

Pravin S. Bhale,[a,b] Hemant V. Chavan,[c,*] Sakharam B. Dongare,[a] Sadanand N. Shringare,[a] Yoginath B. Mule,[a] Samadhan S. Nagane,[d] Babasaheb P. Bandgar[a,*]

[a]*Medicinal Chemistry Research Laboratory, School of Chemical Sciences, Solapur University, Solapur-413 255, Maharashtra, India.*

[b]*Department of Chemistry, Yashwantrao Chavan Mahavidyalaya, Tuljapur, Dist-Osmanabad-413 601, Maharashtra, India.*

[c]*Department of Chemistry, A.S.P. College, Devrukh, Dist-Ratnagiri-415 804, Maharashtra, India.*

[d]*Polymer and Advanced Materials Laboratory, National Chemical Laboratory, Pune-411008, Maharashtra, India.*

Address for correspondence: Email-hemantchavan.sus@rediffmail.com

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ABSTRACT

In the present investigation, synthesis of a series of extended conjugated δ-chloro-α-cyano substituted indolyl chalcones (**5a-p**) was accomplished by reacting 3-cyanoacetylindole **2** with 3-chloro-3-phenyl-propenal **4** in the presence of piperidine. The structural interpretations of newly synthesized compounds were based on chemical and spectroscopic evidences. Anti-tumor evaluation of the synthesized compounds *in vitro* against MCF-7 (breast carcinoma) cell line revealed that they possess high anti-tumor activities. Among them, compound **5e** and **5a** demonstrated excellent activity against breast carcinoma (GI_{50}< 0.1 and 4 μM respectively) as good as adriamycin (GI_{50} <0.1μM). The compounds were also screened against the normal Vero monkey cell line, which showed moderate selectivity against inhibition of cancer cells. The effect of extended conjugation on activity authenticated by comparing activity profile of compound **5a**, **5i** and **5m** with their simple analogues. Among the synthesized compounds, **5i** and **5l** were found to be active anti-inflammatory agents in addition to having noteworthy antioxidant potential. These results suggest the possible use of these compounds for the design and development of novel anti-breast cancer agents.

Key words: Indole chalcones, extended conjugation, cytotoxicity, anti-inflammatory, antioxidant

Cancer is one of the leading causes of death in the society.[1] Cancer is a multifactor disease with superfluous and robust biological network.[2] It is a family of disease, which despite five decades of intense research still affects 1 in 3 people during the course of their live.[3] Breast cancer is the most common invasive cancer in female worldwide. It accounts for 16% of all female cancers and 22.9% of invasive cancers in women, 18.2% of all cancer deaths worldwide including both in males and females are from breast cancer. Breast cancer rates are much higher in developed nations compared to developing ones.[4] Various kinds of treatments are available for breast cancer, such as chemotherapy, radiotherapy and hormone therapy. [5] Recent treatment strategies for cancer have limited efficacy. It may require treatment with compounds that could target multiple intracellular compounds. Nowaday, cancer therapy interfering with a single biological molecule or pathway has been successfully utilized for the treatment in clinics.[6]

When a cell turns cancerous they develop into tumors, which produce various pro-inflammatory and inflammatory cytokines and chemokines that attract leukocytes to the site of growth. The leukocyte in turn produce and assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine and cystine proteases, matrix metallo proteinases (MMPs) and soluble mediators of cell killing such as tumor necrosis factor-alpha (TNF-α), interleukins and interferons (IFNs). All these result in inflammation. The leukocyte then tries to kill the tumor cells to regulate the inflammation. When they are not successful it results in chronic inflammation and progression of tumors. The development of tumors thus depends on the balance between pro and anti-inflammatory cytokines. Taking into consideration the importance of inflammation in cancer development, it can be possible to minimize the risk of cancer development by using anti-inflammatory agents.

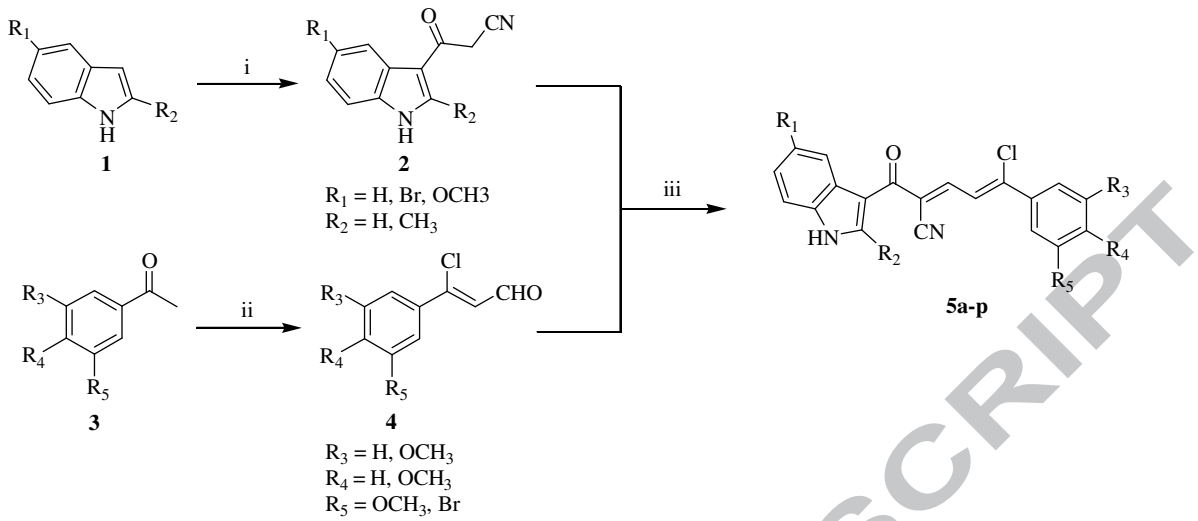
Chalconesconsist of a 1,3-diphenyl-2-propen-1-one framework have received a great deal of attention due to their relatively simple structures and ample variety of pharmacological activities including anticancer, anti-inflammatory, antioxidant, antimicrobial, analgesic, antipyretic, antimalarial, antileishmanicidaland anti-allergic activities.[7] These activities are largely attributed due to the unsaturated ketone moiety.[8] Moreover, introduction of massive substituents on two aryl rings also a subject of interest because it leads to useful structure-activity relationship (SAR) conclusions and thus helps to synthesize pharmacologically active chalcones.[9] The literature survey revealed that very little attempts were made towards the synthesis of diversely substituted extended conjugated chalconesand to verify effects on the biological activity. Extended conjugation and a high degree of electrophilicity associated with the scaffold play a vital role in the activity of

suchclass of compounds.[10] The complete delocalization of π-electrons on both the benzene rings makes these scaffolds more susceptible in undergoing electron transfer reactions that may be attributed to their superior antioxidant activity. Recently, our research group reported chloro substituted extended conjugated chalcones as potent anticancer, anti-inflammatory and antimicrobial agents.[11]

The indole skeleton is one of the most attractive frameworks with a wide range of biological and pharmacological activities. [12] This physiologically important nucleus is abundantly found in therapeutic agents as well as in natural products. [13] Many indole derivatives reported as potent breast cancer agents, such as aplysinopsin analogs, which are indole-derived marine natural products [14] and indole-3-carbinols. [15] Martel-Frachet and coworkers reported mono- and bis-indolylchalcones as potent cytotoxic agents against bladder carcinoma.[16] Lawrence et al. have reported a series of α-substituted chalconesposses promising antiproliferative activity against different cancer cell lines. [17] Tarleton et al. reported (z)-2-(3,4-dichlorophenyl)-3-(4-nitrophenyl)acrylonitrile shows significant cytotoxicity against human breast cancer cell line, MCF-7 (GI_{50}=0.127±0.043μM).[18] More recently, α-cyano substituted indolylchalcones have found to possess potent anticancer potential.[19]

Based upon these observations and in continuation of our ongoing research programmeon the developmentof novel anticancerand anti-inflammatory agents,[20] herein we synthesizeda diverse library of extended conjugated δ-chloro-α-cyanosubstitutedindolylchalcones as potent anti breast cancer, anti-inflammatory and antioxidant agents.

In the present study, synthesis of desired extended conjugated δ-chloro-α-cyano substituted indolylchalcones (**5a-p**) achieved by the Knoevenagel condensation of 3-cyanoacetylindoles **2** with substituted 3-chloro-3-phenyl-propenals **4** in the presence of piperidine in ethanol (**Scheme 1**). [21] The starting compounds for the synthesis of title compounds, namely 3-cyanoacetylindoles**2** synthesized in good yields from the reaction of substituted indoles **1** with cyanoacetic acid in presence of acetic anhydride.[22] On the other hand, 3-chloro-3-phenyl-propenals **4** were synthesized using the method described in the literature with minor modifications. [23] The products obtained were purified by column chromatography using silica gel mesh size, 100–200 and elution with 10% ethyl acetate in hexane. The structures of target molecules were analyzed by IR, ^{1}HNMR, ^{13}CNMR and HRMS.



Scheme 1: Synthesis of extended conjugated indolyl chalcones

Reagents and conditions: i) $CNCH_2COOH$, $(CH_3CO)_2O$, Reflux ii) DMF-$POCl_3$ iii) Piperidine, Ethanol, Reflux 1-4 h

All the synthesized extended indolylchalcones (**5a-p**) were evaluated for their *in vitro* cytotoxic potencies in human breast cancer cell line MCF-7 and normal Vero monkey cell line by employing the sulforhodamine B (SRB) assay method.[24,25] Adriamycin is one of the most effective anticancer agents used as reference drug. Three parameters such as GI_{50}, TGI and LC_{50} were determined during the screening process and the results summarized in **Table 1**.

The GI_{50} values (growth inhibitory activity) refer to the drug concentration that produces a 50% reduction of cellular growth compared with the untreated control cells. The TGI (cytostatic activity) and LC_{50} (cytotoxic activity) values refer to the drug concentration required for total growth inhibition and killing 50% of the cells, respectively. GI_{50} values used to classify a compound's activity as follows: inactive, >100 µM; moderate, between >10 and <100 µM; and active, <10 µM.

It is worth mentioning that most of the compounds were significantly cytotoxic against MCF-7 compared to the standard drug adriamycin, with the concentration of the drug that produced 50% inhibition of cell growth (GI_{50}). Compound **5e** and **5a** exhibited potent activity (GI_{50} = <0.1 and 4 µM respectively) against the MCF-7 cell line which was almost as good as that of adriamycin (GI_{50} = <0.1 µM). Compounds **5f**, **5i**, **5j**, **5m**, **5b**, **5n** and **5c** also exhibited good cytotoxicity (GI_{50} = 29.64 - 47.30 µM). On the other hand, all other compounds showedweak cytotoxicity (GI_{50} = 56.74 - 100 µM) against MCF-7 cell line.

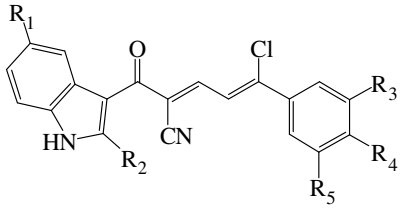
A comparison of the TGI concentrations of the compounds with adriamycin was also done. Most of the compounds were inactive and a few exhibited weak activity against the MCF-7 cell line. Compounds **5h** (TGI = 73.75 μM), **5d** (TGI = 81.5 μM), **5e** (TGI = 83.7 μM), **5f** (TGI = 92.97 μM), **5a** (TGI = 94.3 μM) and **5i** (TGI = 96.5) exhibited weak activity against MCF-7 cell line. All the other compounds found inactive (TGI >100 μM) as compared to standard drug adriamycin.

The LC_{50} concentrations of the compounds were compared to that of adriamycin to get an indication of the cytotoxic effects of these compounds against the MCF-7 cell line. The compounds were inactive (LC_{50} >100 μM) like adriamycin (LC_{50} = 97.1 μM) against the MCF-7 cell line.

Structure activity relationship (SAR) study revealed that introduction of methoxy groups at 3,4,5-positions of benzene ring increase in activity was observed, whereas, decrease in activity was observed with bromo substitution.The presence of methyl group at 2-position of indole also enhanced activity to greater extent. Compound **5e** acquiring methyl group at 2-position of indole ring exhibited potent activity (GI_{50} = <0.1 μM, TGI = 83.7 μM) against MCF-7 cell line.

To confirm the effect of extended conjugation on cytotoxic potential, we have prepared three simple α-cyano substituted indolyl chalcone analogues of compound **5a**, **5i** and **5m** by reacting suitably substituted 3-cyanoacetylindole with 3,4,5-trimethoxybenzaldehyde by refluxing in ethanol in the presence of piperidine. These simple chalcone analogues were also subjected for cytotoxicity evaluation against MCF-7 cancer cell line. The comparison of GI_{50} values for **5a**, **5i** and **5m** and their simple chalcone analogues reveals that presence of extended conjugation greatly increases the cytotoxic potential of compound **5a**, **5i** and **5m** (**Figure 1**). Significant increase in activity of compound **5e** (Indole ring bearing 2-methyl group**)** compared to compound **5a** (absence of methyl group) authenticates the presence of methyl group at 2-position of indole moiety is essential for activity (**Figure 1**).

Table 1. In vitro anticancer screening of extended conjugated indolyl chalcones (**5a-p**) against human breast cancer cell line MCF-7.[a]



Compound	R_1	R_2	R_3	R_4	R_5	MCF-7			Vero (Normal)		
						LC_{50}[b]	TGI[c]	GI_{50}[d]	LC_{50}	TGI	GI_{50}
5a	H	H	OCH_3	OCH_3	OCH_3	>100	94.3	**4.0**	54.2	35.9	16.2
5b	H	H	OCH_3	OCH_3	H	>100	>100	44.20	NT	NT	NT
5c	H	H	H	OCH_3	H	>100	>100	47.30	NT	NT	NT
5d	H	H	H	Br	H	>100	81.5	72.50	NT	NT	NT
5e	H	CH_3	OCH_3	OCH_3	OCH_3	>100	83.7	**<0.1**	68.3	42.6	12.4
5f	H	CH_3	OCH_3	OCH_3	H	>100	92.97	29.64	65.9	41.1	11.8
5g	H	CH_3	H	OCH_3	H	>100	>100	56.74	NT	NT	NT
5h	H	CH_3	H	Br	H	>100	73.75	73.91	NT	NT	NT
5i	Br	H	OCH_3	OCH_3	OCH_3	>100	96.5	30.84	41.5	22.1	7.6
5j	Br	H	OCH_3	OCH_3	H	>100	>100	31.90	51.8	38.4	8.2
5k	Br	H	H	OCH_3	H	>100	>100	80.30	NT	NT	NT
5l	Br	H	H	Br	H	>100	>100	>100	NT	NT	NT
5m	OCH_3	H	OCH_3	OCH_3	OCH_3	>100	>100	39.20	45.2	12.1	9.5
5n	OCH_3	H	OCH_3	OCH_3	H	>100	>100	44.90	NT	NT	NT
5o	OCH_3	H	H	OCH_3	H	>100	>100	87.10	NT	NT	NT
5p	OCH_3	H	H	Br	H	>100	>100	79.86	NT	NT	NT
Adriamycin						**97.1**	**11.0**	**<0.1**	**52.6**	**>10**	**19**

[a] Concentrations in μM; [b] Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) calculated from $[(Ti - Tz)/Tz]x100 = -50$; [c] Drug concentration resulting in total growth inhibition (TGI) will calculated from $Ti = Tz$; [d] Growth inhibition of 50% (GI_{50}) calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$; NT = Not.tested.

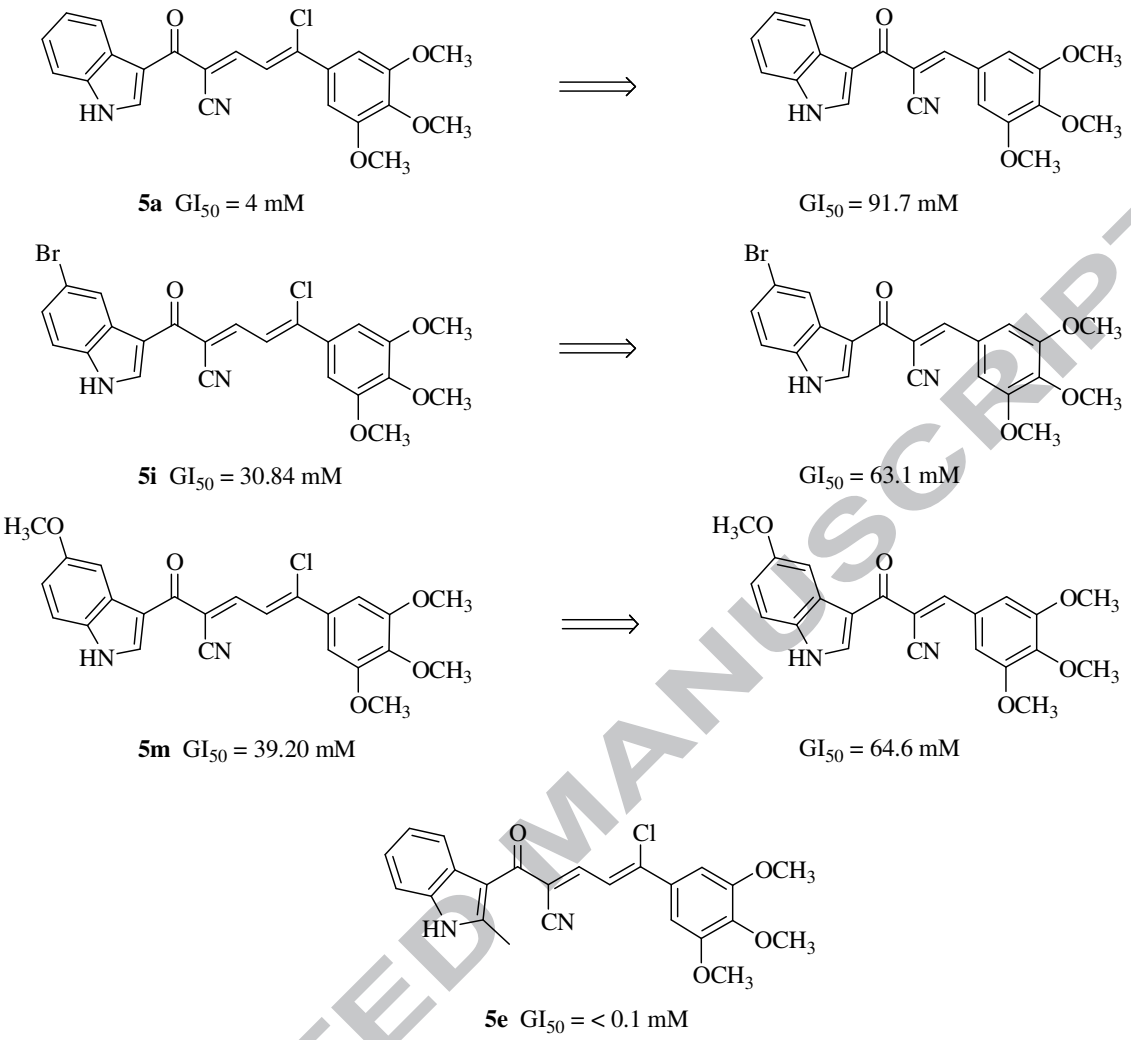


Figure 1. Effect of extended conjugation on cytotoxicity potential of compound **5a**, **5i** and **5m**

Most of the reported drugs affect the normal cell growth, which serves to be a major disadvantage in the progress of anticancer drug development. Therefore, we have ensured the selectivity of some active compounds by *in vitro* screening against the normal Vero Monkey cell line. This cellular level normal screening results help to reveal the safety profile of active compounds. This cytotoxicity study revealed that the GI_{50} values of compound **5a**, **5e, 5f, 5i, 5j** and **5m** are 16.2, 12.4, 11.8, 7.6, 8.2 and 9.5 μM respectively (Table 1). These results illustrate that the synthesized methyl substituted extended conjugated indolylchalcones**5a** and **5e** may have drug like properties at cellular level. This new lead compounds also show moderate selectivity against cancer lines over normal cell line.

Denaturation of proteins is a well-documented cause of inflammation. In the present study, the *in vitro* anti-inflammatory effect of synthesized compounds was evaluated against denaturation of egg albumin[26] and results are summarized in **Table 3**. Compound **5i** and **5l** showed significant inhibition (90.82%) compared to the Diclofenac sodium, a standard anti-inflammation drug (90.21%) at 1mM concentration. All the other compounds were also found to be effective in inhibiting heat induced albumin denaturation (78.23-88.07%) except compound **5d** and **5j**.

Table 3. Effect of extended conjugated chalcones on heat induced protein denaturation

Entry	% inhibition (1 mM)
5a	86.23
5b	85.32
5c	80.23
5d	38.53
5e	80.73
5f	79.81
5g	83.10
5h	85.32
5i	90.82
5j	21.10
5k	78.23
5l	90.82
5m	84.40
5n	87.15
5o	79.53
5p	88.07
Diclofenac sodium	90.21

Free radicals especially the reactive oxygen species (ROS) are proposed to be the key players in the pathophysiological mechanisms associated with various inflammatory disorders.[27] These radicals reacts indiscriminately with almost every type of biomolecules found in living cell and may deviate the cells from its normal physiological functions.[28] Antioxidants are the compounds capable of scavenging the free radicals; for this antioxidant therapy is one of the recent options.[29] Taking into the account of multifactorial character of

oxidative stress; involved in many pathological states, the entire series of extended indolylchalcones (**5a-p**) were evaluated for their direct scavenging activity against a variety of reactive oxygen and nitrogen species such as 2,2-diphenyl-2-picrylhydrazyl (DPPH),[30] nitric oxide (NO)[31] andsuperoxide (SOR).[32,33] Free radical scavenging activity was measured in terms of percent inhibition and results are presented in **Table 4**. All the synthesized compounds have shown good to excellent scavenging activity against DPPH, NO and SOR radicals (**Fig. 2**). Most of the synthesized compounds possesses excellent NO radical scavenging activity. Compounds **5h, 5c, 5d, 5p, 5f, 5b, 5e, 5g** and **5a** have shown excellent NO radical scavenging activity (78.33-73.33%), whereas, all other compounds showed good activity (68.33-63.66%). The compounds **5m** and **5p** showed good DPPH free radical scavenging activity (40–46%). All other compounds showed moderate DPPH radical scavenging activity (11-34%). Compounds **5l, 5j** and **5h** were found to possess significant SOR scavenging activity (83.63-69.09%) as compared to standard ascorbic acid (AA) (74.07%). All other compounds were moderate SOR scavengers (66.37–50%).

Table 4. *In vitro* anti-oxidant activity of extended conjugated indolyl chalcones (**5a-p**)

Entry	% inhibition at 1mM		
	DPPH	**NO**	**SOR**
5a	21.05	**73.33**	59.09
5b	15.78	**75.00**	63.63
5c	23.36	**76.88**	61.32
5d	18.42	**76.66**	65.45
5e	14.47	**74.16**	58.18
5f	22.36	**75.83**	66.36
5g	34.17	**74.00**	63.07
5h	15.78	**78.33**	**69.09**
5i	**26.31**	36.66	50.00
5j	11.84	57.50	**80.90**
5k	15.60	56.18	62.17
5l	15.78	67.50	**83.63**
5m	**46.05**	68.33	50.00
5n	34.21	**74.16**	58.18
5o	16.70	41.63	60.72
5p	**40.78**	**76.66**	54.54
AA	44.18	42.63	74.07

Standard: AA = Ascorbic acid; data represent mean of two replicates.

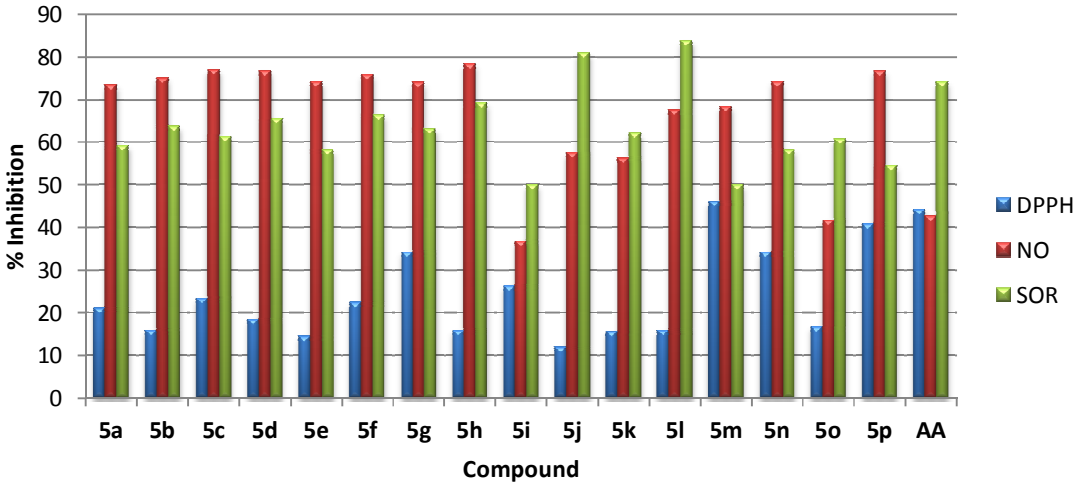


Figure 2. Antioxidant activity profile of synthesized compounds

In summary, we rationally designed and synthesized a series of extended conjugated δ-chloro-α-cyano substituted indolyl chalcone derivatives and in vitro evaluated them for potential cytotoxic activity against breast carcinoma (MCF-7) Most of the compounds under investigation exhibited significant antitumor activities. Among them, compound **5e** and **5a** show higher activity against breast carcinoma as good as adriamycin. These results further support its safety margin by studying the activity on normal Vero monkey cell line. In general, the extended conjugation and substitution of alkyl group at 2-position of indole leads to increase the cytotoxic activity. Anti-inflammatory and Antioxidant potential of synthesized compounds were also evaluated. All the compounds found to possess marked anti-inflammatory potential by effectively inhibiting the heat induced albumin denaturation. Most of the compounds exhibited significant NO and SOR scavenging activity, whereas moderate DPPH radical scavenging activity. The present investigation has thus provides impetus for the design and development of more potent chalcone derivatives as anticancer leads.

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21. *General procedure for the synthesis of extended conjugated indolylchalcones (**5a-p**):*To a mixture of 3-(1H-indol-3-yl)-3-oxopropanenitrile**2** (1 mmol) in ethanol (15 ml) was added piperidine (0.3 ml) and stirred for 5 min. Then, added the purified β-chlorovinyl aldehyde **4** (1 mmol) and this mixture was heated to 70^{o}C for 1-4 h. After completion of reaction (monitored by TLC), reaction mixture was poured over crushed ice and acidified with acetic acid. The precipitated solid was filtered, washed with water and oven dried. It was column purified by column chromatography using silica gel mesh size, 100–200 and elution with 10% ethyl acetate in hexane.
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24. *Procedure of the SRB-assay:* Tumor cells (human breast cancer cell line MCF-7) were grown in tissue culture flasks in growth medium (RPMI-1640 with 2 mM glutamine, pH 7.4, 10% fetal calf serum, 100 mg/ml streptomycin, and 100 units/ml penicillin) at 37^{o}C under the atmosphere of 5% CO_2 and 95% relative humidity employing a CO_2 incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and placed in growth medium. The cells with more than 97% viability (trypan blue exclusion) were used for cytotoxicity studies. An aliquot of 100 ml of cells were transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for one day at 37^{o}C in a CO_2 incubator as mentioned above. The test materials at different concentrations were then added to the wells and cells were further allowed to grow for another 48 h. Suitable blanks and positive controls were also included. Each test was performed in triplicate. The cell growth was stopped by gently layering of 50 ml of 50% trichloroacetic acid. The plates were incubated at 4^{o}C for an hour to fix the cells attached to the bottom of the wells. Liquids of all the wells were gently pipette out and discarded. The plates were washed five times with doubly distilled water to remove TCA, growth medium, etc and were air-dried. 100 ml of SRB solution (0.4% in 1% acetic acid) was added to each well and the plates were incubated at ambient temperature for half an hour. The unbound SRB was quickly removed by washing the wells five times with 1% acetic acid. Plates were air dried, tris-buffer (100 ml of 0.01 M, pH 10.4) was added to all the wells and plates were gently stirred for 5 min on a mechanical stirrer. The optical density was measured on ELISA reader at 540 nm. The cell growth at absence of any test material was considered 100% and in turn growth inhibition was calculated. GI_{50} values were determined by regression analysis.

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26. *In vitro anti-inflammatory activity by protein denaturation method:* The reaction mixture (10 ml) consisted of 0.4 ml of egg albumin (from fresh hen's egg), 5.6 ml of phosphate buffered saline (PBS, pH 6.4) and 4 ml of synthetic derivative (1 mM). Similar volume of double-distilled water served as control. Then the mixtures were incubated at (37oC ± 2) in an incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium (1 mM) was used as reference drug and treated similarly for the determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula, % inhibition = 100 x (Vt / Vc - 1); Where, Vt = absorbance of test sample, Vc = absorbance of control.

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30. *DPPH radical scavenging activity:* The ability of compounds **5a-p** to scavenge DPPH radical was assessed using reportedmethod[24] with modification. Briefly, 1 ml of synthesized compounds as 1 mM was mixed with 3.0 ml DPPH (0.5 mmol/L in methanol), the resultant absorbance was recorded at 517 nm after 30 min incubation at 37°C. The percentage of scavenging activity was derived using the following formula, Percentage inhibition (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$; Where, $A_{control}$ is absorbance of DPPH; A_{sample} is absorbance of reaction mixture (DPPH with Sample).

31. *NO radical scavenging activity:* NO radical scavenging activity of compounds **5a-p** was carried out as per the method.[24] NO radicals were generated from sodium nitroprusside solution. One ml of 10 mM sodium nitroprusside was mixed with 1 ml of 1 mM synthetic compounds in phosphate buffer (0.2 M pH 7.4). The mixture was incubated at 25°C for 150 min. After incubation the reaction mixture mixed with 1.0 ml of pre-prepared Griess reagent (1% sulphanilamide, 0.1% napthylethylenediamine dichloride and 2% phosphoric acid). The absorbance was measured at 546 nm and percentage of inhibition was

calculated using the same formula as above. The decreasing absorbance indicates a high nitric oxide scavenging activity.

32. *Superoxide radical (SOR) scavenging assay:* The reaction mixture consisting of 1ml of nitro blue tetrazolium (NBT) solution (156 mM NBT in phosphate buffer, pH 7.4), 1 ml NADH solution (468 mM NADH in phosphate buffer, pH 7.4), and 1ml of synthetic compound (1mM) solution was mixed. The reaction was started by adding 1 ml of phenazine methosulfate (PMS) solution (60 mM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25^{o}C for 5 min and the absorbance was measured at 560 nm against blank sample and compared with standards and percentage of inhibition was calculated using the same formula as above. Decreased absorbance of reaction mixture indicated increased SOR scavenging activity.

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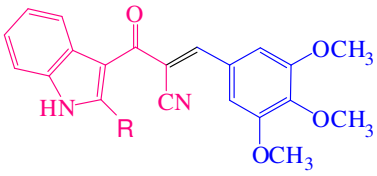
Graphical Abstract

Synthesis of extended conjugated indolyl chalcones as potent anti-breast cancer, anti-inflammatory and antioxidant agents

Pravin S. Bhale,[a,b] Hemant V. Chavan,[c,*] Sakharam B. Dongare,[a] Sadanand N. Shringare,[a] Yoginath B. Mule,[a] Samadhan S. Nagane,[d] Babasaheb P. Bandgar[a,*]



R = H; (**MCF-7**; GI_{50} = **91.7** μM)