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Antiproliferative and antibacterial activity of some *para*-substituted benzylideneacetophenones and establishing their structure activity relationship

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We report here *in-vitro* antiproliferative and antibacterial activity of *para*-substituted benzylideneacetophenones and established their structure activity relationship to optimize *para* position as a *biologically-oriented-synthetic* target for design of small molecule-based future anticancer/antibacterial agents. Among synthesized compounds, 1c exhibits excellent antiproliferative activity against human osteosarcoma cell line (MG-63) compared to 1b and 1a suggesting dimethylamino ($-N(CH_3)_2$) functionality as a better *para*-substituted analogue for *in-future* anticancer agents. Similarly antibacterial screening of the aforesaid compounds against different strains of Gram-negative and Gram-positive bacteria reveals methoxy ($-OCH_3$) rather than dimethylamino ($-N(CH_3)_2$) as a better *para*-substituted functionality on ring B comparatively. From our results, we justify our theory 'lipophilicity affects antibacterial activity'.

Keywords: Antiproliferative, antibacterial assay, benzylideneacetophenone, MTT assay.

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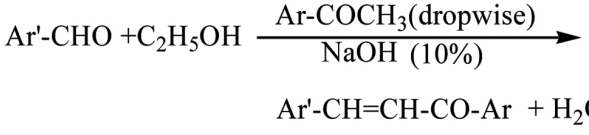
CANCER, a complex disease of genome (DOG) is responsible for intense ill-health globally. In India the disease accounted for 666,563 deaths in 2015 and continues to swing with an estimate of 8 lakhs new cases/year (WHO website)[1]. As per global indices, the disease causes approximately 6-million deaths/year and is estimated to have prevalence of 15-million by 2020. Pathologically, cancer is multi-faceted in origin, involving innumerable factors in its progression and clinically manifests cellular transformation, hyperproliferation, angiogenesis and distal metastasis (Union Internationale Contre Le Cancer)[2]. Despite significant elucidation in carcinogenic molecular mechanism and intensive clinical trials, cancer remains a principal community problem with limited curative interventions. It has been estimated that, by 2030 the universal medical cost for its therapeutic prevention and management may lead up to 458 billion dollars (ref. 3). Numerous obstacles are identified for disease progression and advancement and if uncured, promotes cancer to end-stage. Though chemotherapy is a first line global affirmative with remarkable pharmacological appurtenance, it suffers from limitations such as toxicity, non-selectivity, and tumour acquired resistance. Hence novel anticancer agents with better therapeutic profile and easy to 'reach by all' are the need of the present era[4–6].

Undeniably, antibiotics are a momentous therapeutic invention protecting public health globally. However widespread indiscriminate and continual usage of this miracle-medicine pushes the world ultimately into an astonished era bubbling with antibiotic resistant strains (ARS) such as Carbapenem resistant *Enterobacteriacea*, drug-resistant *Neisseria gonorrhoeae*, multi-drug resistant *Acinetobacter*, drug resistant *Campylobacter*, Fluconazole resistant *Candida*, extended spectrum beta-lactamase producing *Enterobacteriacea*, Vancomycin resistant *Enterococcus*, multidrug resistant *Pseudomonas aeruginosa*, drug resistant non-typhoidal Salmonella, drug resistant *Salmonella typhi*, Methicillin resistant *Staphylococcus aureus*, drug resistant *Staphylococcus pneumonia*, etc.[7–10]. Further, an intentional or unintentional non-compliance of antibiotic regimen owing to bitter taste, high cost, untolerated adverse drug reactions, contra-indication with regular usage medicines, forgetfulness, communication gap between patient and clinician further bulk the basket of resistant bacterial strains[11], causing failure of classical antibiotic therapy. Hence an extensive, in-depth, assertive for search and development of alternate antibacterial agents to contest microbial infection is needed for the present human civilization. Researchers worldwide have dynamically fuelled their attention towards design and development of newer antibiotics as a better deal for in-future emerging bacterial strains.

Benzylideneacetophenone, a distinct class of 1,2-unsaturated carbonyl compound, chemically categorized as 1,3-diaryl-2-propen-1-ones is a key synthetic analogue for yielding a synthetic entity endowed with multiple pharmacological activities[12–22]. Benzylideneacetophenone composed of dual aromatic rings, bridged together with a keto-ethylenic chain (–CO-CH=CH–) enveloping two different functionalities (C=O and C=C), significantly differ in electron distribution, providing the molecule a unique reactivity pattern against electrophilic and nucleophilic reagents, making it an attractive target for chemical modification. Though it occurs naturally, isolation of benzylideneacetophenone (due to enzyme chalcone synthase) is tedious, time-consuming and complicated. The isolation is hardly comparable to synthetic procedures in terms of end ease of synthesis, product yield, purity and overall time consumption.

All the reagents and solvents used in this experiment were acquired from the common store of University Institute of Pharmacy, CSJM University, Kanpur, India. ^{1}H-NMR spectra were recorded by Bruker Avence II 300 Nuclear Magnetic Resonance (NMR) spectrometer. The chemical shifts were recorded in parts per million (ppm) and reported relative to the tetra methyl saline (TMS). Mass spectra were recorded on Waters Q-TOF Premier-HAB213 system in ESI mode. The Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on Perkin Elmer Spectrum version 10.03.06. The melting point was recorded by open capillary method and is uncorrected. Progression of reaction was measured on aluminum plates pre-coated with silica gel-G using chloroform : methanol (9 : 1) as the solvent system and spots were visualized under UV cabinet.

The benzylideneacetophenones were synthesized (Scheme 1) by adopting the following methodology. In a clean ground necked conical flask, a solution of sodium hydroxide (10%) was transferred followed by ethanol (10 ml). The flask was then immersed in an ice-chest and fitted with a sensitive thermometer. Once the reading of the thermometer was fixed at 0°C, aromatic aldehyde (0.01 M) was transferred at once and stirring was initiated. Immediately, distilled acetophenone (0.01 M) was slowly and steadily transferred into the reaction vessel with stirring, while maintaining the overall reaction temperature not greater than 15°C. Once exhausted, the ice-chest was replaced by a water-bath (25°C) and the stirring was resumed for the next 3 h. The crude product obtained was filtered off, washed thoroughly, and recrystallized from ethanol. The progression of reaction and purity of product were assessed from thin layer chromatography (TLC) employing chloroform : methanol solvent system in ratio 9 : 1.



Scheme 1.

Benzylideneacetophenone (**1a**): Light yellowish colour solid; $C_{15}H_{12}O$: m.p.: 57–59; yield 82%; IR (KBr) cm^{-1}: 3030 (CH-Ar), 1558 (C=O), 1598 (C=C); NMR (δ, ppm): 7.7 (d 1Hα) 8.0 (1Hβ) 7.4–7.9 (m, 10H-Ar-H); Mass ESI (m/e): 208.

4-methoxbenzylideneacetophenone (**1b**): White-yellowish colour solid; $C_{16}H_{14}O_2$: m.p.: 77–79; yield 89%; IR (KBr) cm^{-1}: 2955 (CH-Ar), 1658 (C=O), 1598 (C=C), 1111 (–OCH_3); NMR (δ, ppm): 7 (d 1Hα) 8.1 (d, 1Hβ) 6.4–8.1 (m, 9H-Ar-H), 3.6 (s, 3H, –OCH_3); Mass ESI (m/e): 238.

4-dimethylaminobenzylideneacetophenone (**1c**): Red colour solid; $C_{17}H_{17}NO$: m.p.: 110–112; yield 93%; IR (KBr) cm^{-1}: 2906 (CH-Ar), 1661 (C=O), 1599 (C=C), 1313 (CN); NMR (δ, ppm): 6.9 (d, 1Hα) 8.0 (d, 1Hβ) 7.0–7.5 (m, 9H-Ar-H), 2.6 (s, 6H, –$N(CH_3)_2$; Mass ESI (m/e): 251.

Human osteosarcoma (MG-63) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Himedia) with 4.0 mM L-glutamine, 1.5 g/l $NaHCO_3$, 1.0 mM sodium pyruvate, 1.0% penicillin and streptomycin solution and supplemented to contain 10% (v/v) fetal calf serum (Himedia). Cells were grown at 37°C, 5% CO_2 in a humidified air.

The cytotoxicity of synthesized compounds was evaluated on human osteosarcoma cell line (MG-63) by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[23]. Theoretically, the assay provides a direct relationship between cell viability and colour formation (absorbance). The human osteosarcoma (MG-63) cells were seeded at an initial density of 1×10^4 cells/100 µl complete culture media in 96-well plate and incubated for 24 h at 37°C in humidified, 5% CO_2 atmosphere. Cells were further treated with different concentrations (10–200 µM) of synthesized compounds (**1a–1c**) to elucidate their antiproliferative profile. After 24 h of treatment, morphological changes were observed by using phase contrast microscope. The medium was removed and the cells were incubated further with 20 µl of MTT (5 mg/ml in PBS) in fresh medium for 4 h at 37°C. Finally, the formazan crystals formed by mitochondrial reduction of MTT were solubilized in 200 µl DMSO and absorbance was recorded at 570 nm after 10 min incubation with the aid of a microplate reader (BIO RAD model 680). All the experiments were performed in triplicates and the absorbance of control cells was taken as 100% viability. Percent of inhibition of cytotoxicity was calculated as a fraction of control. The percentage cell viability was calculated using the formula

$$\% \text{ Cell viability} = \frac{\text{Treated absorbance}}{\text{Controlled absorbance}} \times 100.$$

The pure cultures of experimental bacterial strains were obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh. The bacterial strains were further cultured and sub-cultured according to the standard, well reported, authenticated protocol.

The *in vitro* antibacterial assay of the aforementioned synthesized compounds was performed by classical disc diffusion method (DDM). The result of the same was evaluated in terms of zone of inhibition (ZOI) recorded in mm depicting extent of antibacterial property; comparing against standard (chloramphenicol). The bacterial suspensions of the test culture were prepared from 24 h old bacterial culture. The method comprises puncturing the discs of equal sizes from Whatman filter paper no. 1 and soaking it from a solution of compound under test as well as standard drug solution. The stock solution of compounds was prepared in 1% DMSO (including control) to get a final concentration of 500 and 1000 µg/ml. The comparative reference standard for bacterial strain under test was prepared separately at similar concentration of chloramphenicol as mentioned above, however, this time in sterile-water. The culture plates were incubated at 37°C for 24 h. The antibacterial activity was measured in terms of zone of inhibition in mm. All the experiments were performed in triplicate.

Benzylidene acetophenones (**1a–1c**) were synthesized in good yield (82–93%) and their structures confirmed by suitable spectrometric analysis, viz. IR, ^{P}NMR, and mass spectra. Furthermore *in vitro* activity of the aforesaid compounds (**1a–1c**) were assayed via standard protocol.

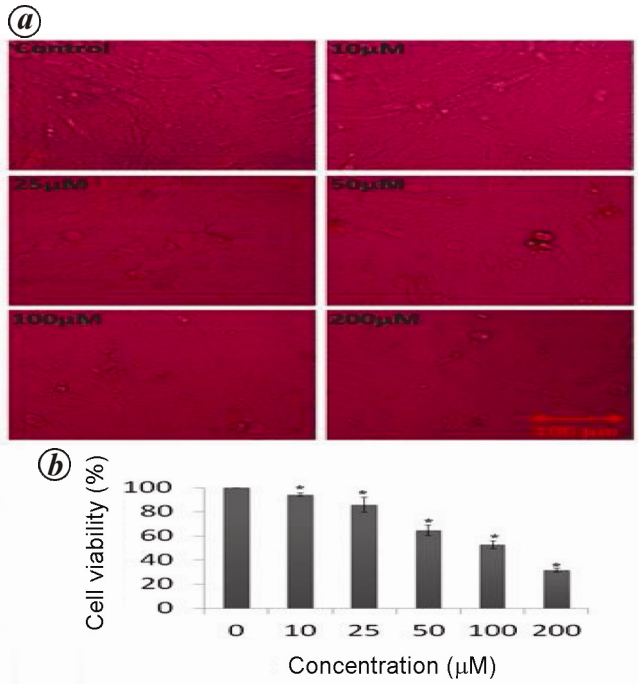


Figure 1. Antiproliferative activity of compound **1a** against osteosarcoma (MG-63). ***a***, Morphological analysis of live and dead cell of MG-63 cells treated with 10, 25, 50, 100 and 200 µM of compound **1a** as compared to control. ***b***, % cell viability of MG-63 cells by a MTT assay treated with 10–200 µM of Compound **1a** for the period of 24 h. Values were obtained from three independent experiments and expressed as mean ± SEM. **P*0 < 0.05 as compared with control.

The dose-dependent inhibitions in percentage cell viability of compounds were observed at differential concentrations (10–200 μM) and the results compared with control (Figures 1–3). The compound **1c** was most cytotoxic, followed by **1b**. Finally **1a** was the least cytotoxic against human osteosarcoma cell lines (MG-63). The morphological features of cells by phase contrast microscopy reveal typical features of apoptosis such as cell shrinkage, condensation and formation of cytoplasmic blebs[24], suggesting antiproliferative activity of benzylideneacetophenones. Furthermore, the percentage cell viability count at highest concentration among compounds indicates **1c** is having the lowest value compared to **1b** and **1a**. Likewise percentage cell viability count of the same compounds at preliminary dose indicates 91.77, 93.01 and 94.23 with respect to control. Thus the overall sequence of toxicity among compounds was found to be **1c** > **1b** > **1a** (Table 1).

The antibacterial activity of benzylideneacetophenones (**1a–1c**) was determined by disc diffusion method (DDM) at two different concentrations (500 and 1000 μg/ml) against *E. coli*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* against chloramphenicol as standard, which reveals their moderate to mild antibacterial characteristics.

It has been found that a strain of Gram-positive bacteria (*Staphylococcus aureus*) is more susceptible towards synthesized compounds (**1a–1c**), compared to Gram-negative strains (*E. coli*, *P. aeroginosa*). Among screened compounds, **1b** shows superior activity against *Staphylococcus aureus* with an overall percentage inhibition of 23 and 41 at both (500–1000 μg/ml) concentrations respectively. However at similar concentration, compound **1c** exhibits moderate and **1a** shows mild antibacterial activity against the same strain compared to standard (Table 2). Further our data reveals that most of the synthesized compounds are inactive against Gram-negative bacterial strains. However compounds **1b** and **1c** exhibit minimal potency against *E. coli* and *P. aeroginosa* at highest concentration of 1000 μg/ml which is found to be 12 and 09 respectively compared to standard. The reason for such difference in activity between Gram-positive and Gram-negative bacterial strains was assumed due to their distinct membranous composition and natural unique structural framework along with differential intake of compounds being under test. In summary,

Table 1. Percentage inhibition in cell growth at different concentrations of compounds **1a–c**

Concentration (μM)	Compounds and % cell viability		
	1a	**1b**	**1c**
10	94.23	93.01	91.77
25	85.65	77.92	78.98
50	64.87	63.69	53.66
100	52.76	46.36	38.76
200	31.64	30.83	24.90

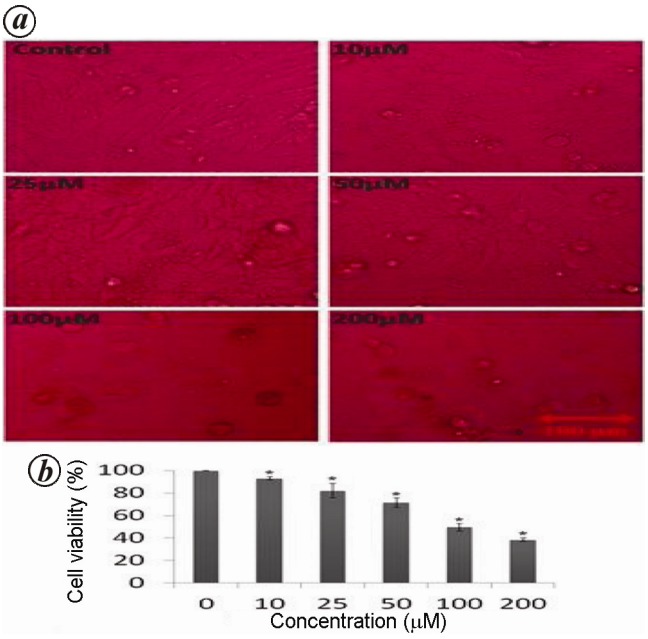


Figure 2. Antiproliferative activity of compound **1b** against osteosarcoma (MG-63). ***a***, Morphological analysis of live and dead cell of MG-63 cells treated with 10, 25, 50, 100 and 200 μM of Compound **1b** as compared to control. ***b***, % cell viability of MG-63 Cells by a MTT assay treated with 10–200 μM of Compound **1b** for the period of 24 h. Values were obtained from three independent experiments and expressed as mean ± SEM. **P*0 < 0.05 as compared with control.

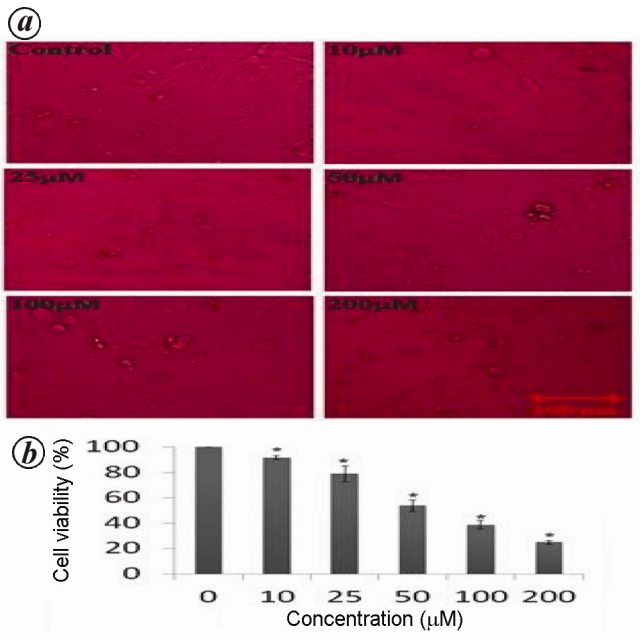


Figure 3. Antiproliferative activity of compound **1c** against osteosarcoma (MG-63). ***a***, Morphological analysis of live and dead cell of MG-63 cells treated with 10, 25, 50, 100 and 200 μM of compound **1c** as compared to control. ***b***, % cell viability of MG-63 cells by a MTT assay treated with 10–200 μM of compound **1c** for the period of 24 h. Values were obtained from three independent experiments and expressed as mean ± SEM. **P*0 < 0.05 as compared with control.

Table 2. Percentage inhibition in bacterial growth at different concentrations of compounds **1a–c**

	E. coli (G–)		*Pseudomonas aeroginosa* (G–)		*Staphylococcus aureus* (G+)	
Bact. Str. Compd.	500 µg/ml	1000 µg/ml	500 µg/ml	1000 µg/ml	500 µg/ml	1000 µg/ml
1a	–	–	–	–	19	26
1b	–	12	–	–	23	41
1c	–	–	–	09	21	31
Standard	40	59	63	76	39	52

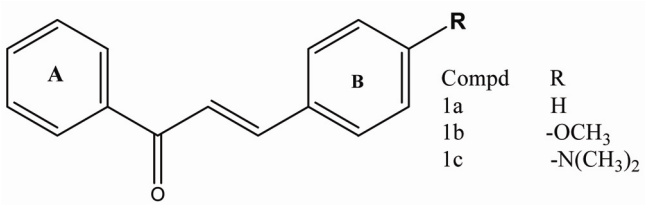


Figure 4. Core structure of **1a–c**.

Staphylococcus aureus is most susceptible and *Pseudomonas aeroginosa* are the most resistant strains among screened compounds.

All the three benzylideneacetophenones share common backbone bearing dual aromatic rings **A** and **B** separated by single two-carbon atom bridge enveloping a keto-ethylenic (C=C-C=O) moiety. The difference amongst them lies at the 4th position (*para* position) on ring **B**; compound **1a** bears hydrogen, **1b** retains methoxy, and **1c** a dimethylamino functionality (Figure 4). Though similar in core structure, the compounds differ substantially in physiochemical characteristics such as melting point, solubility and physical appearance owing to presence of different functionalities at *para*-position on ring **B**. In terms of *in vitro* biological prospective, compounds substituted at *para* position (**1b** and **1c**) show better antiproliferative as well as antibacterial properties compared to their unsubstituted analogue (**1a**). The reason for the same was assumed to be due to presence of extra functionality on aromatic ring (**B**) which may assist in the formation of newer chemical bonds with amino acid residue of protein or simultaneously affect their lipophilic characteristics, thus potentiating biological activity. Among screened compounds, **1c** exhibits better antiproliferative activity against human osteosarcoma cell line (MG-63) at five different concentrations (10–200 µM) compared to its close conger **1b** and unsubstituted derivative **1a** suggesting better antiproliferative characteristic of dimethylamino ($-N(CH_3)_2$) functionality compared to methoxy ($-OCH_3$) group. However when the same compounds (**1a–1c**) were screened for their *in-vitro* antibacterial activity, the opposite results were obtained indicating better antibacterial property of compound **1b** bearing methoxy ($-OCH_3$) group over compound **1c** holding dimethylamino ($-N(CH_3)_2$) functionality at *para*-position on the same ring. This confirms assumption 'high lipophilicity-enhance-antibacterial activity'.

From the above discussion it is clear that *para*-substituted benzylideneacetophenones **1b** and **1c** exhibit better *in vitro* biological activities (antiproliferative and antibacterial activity) compared to non-substituted analogue **1a** against human osteosarcoma cell line (MG-63) and Gram-positive bacterial strain *Staphylococcus aureus*, possibly suggesting it as a candidate molecule for *in future* anticancer and antibacterial agent. It is further considered that suitable derivatization of core structure with different functional groups at *para* position on ring B, ring A, and both would yield a drug with better therapeutic profile, selectivity, specificity, and 'reach by all' for human civilization.

Conflict of interest: The authors declare no conflict of interest.

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