Med Chem Res (2014) 23:3941–3952
DOI 10.1007/s00044-014-0973-5

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Hybrid probes of aromatic amine and barbituric acid: highly promising leads for anti-bacterial, anti-fungal and anti-cancer activities

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Received: 20 November 2013 / Accepted: 18 February 2014 / Published online: 6 March 2014

Abstract Today, cancer and resistant microbes remain one of the most deadly diseases in the world. In search of novel anti-cancer and anti-microbial probes, a series of newly hybrid molecules is synthesized by combining the structural features of aromatic amines and barbituric acid, using the concept of green chemistry. This approach was accomplished efficiently using water as the greener solvent and in the absence of catalyst to give the corresponding adducts. All newly synthesized compounds were characterized by spectral analysis FT-IR, ^{1}H NMR, ^{13}C NMR, HMBC and Elemental Analysis. Evaluations of these probes over four human cancer cell lines (*Breast adenocarcinoma cancer cell line* MCF-7, *Non-small cell lung cancer cell line* NCI-H460, *CNS cancer cell line* SF-268 and *fibroblast cancer cell line* WI-38), anti-microbial activity against five bacterial strains (*S. pyogenes* MTCC 442 and *S. aureus* MTCC 96 as the gram positive, *E. coli* MTCC 443, *P. aeruginosa* MTCC 424 and *K. pneumoniae* MTCC 109 as the gram negative) and four fungal strains (*C. albicans* MTCC 227, *A. clavatus* MTCC 1323, *T. rubrum* MTCC 296 and *Penicillium wild strain*). Out of set of nineteen probes, three probes show significant anti-cancer activities against MCF-7, NCI-H460 and SF-268, whereas sixteen probes exhibit potent anti-tumour activity against WI-38 cell lines. Within anti-microbial bioassay, three molecules exhibited significant activity against both the gram-positive as well as gram-negative bacteria, whereas two compounds showed highly potent activity against *T. rubrum* fungi, while three molecules were found to be equipotent against *T. rubrum* as a fungal strain.

Keywords Green chemistry · *N*-formylation · Knoevenagel condensation · Anti-bacterial · Anti-fungal · Cytotoxicity

Introduction

In recent years, cancer and resistant microbes continuing to be a major health problem in both developed and developing countries. Several anti-cancer agents including taxol, vinblastine, vincristine, etoposide, camptothecin and its derivatives (topotecan and irinotecan), mitoxantrone, 5-fluorouracil, indomethacin (Singh *et al*., 2009), etc., are in clinical use all over the world. However, these drugs suffer from various side effects like low blood pressure, bone marrow suppression, gastrointestinal toxicity, constipation and hair loss. Therefore, the lack of effective chemotherapy of cancer is continuously inciting the scientific community to explore new chemical entities for the effective and safe cure of cancer. The major role of small chemical motifs, such as nucleobases, amino acids and glucose etc., owing to significant acceptability of their synthetic analogues in the biological systems may be the

Electronic supplementary material The online version of this article (doi:10.1007/s00044-014-0973-5) contains supplementary material, which is available to authorized users.

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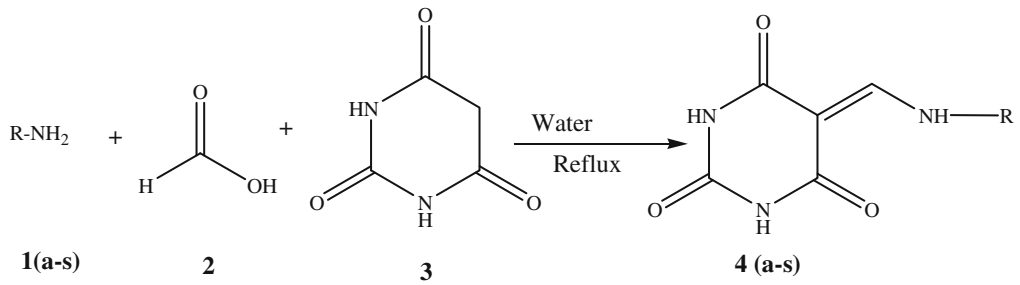
reason for the design and development of about 80 % of the drugs based on small organic molecules (Meunier, 2008). The biological properties of barbiturates include: anti-convulsant (Nicoll and Wojtowicz, 1980), anti-hypnotic (Huang and Barker, 1980), anti-cancer (Dhorajiya *et al*., 2014), anti-inflammatory Goodman and Gilman, 1991) and antimicrobial agents (Sundberg, 1996; Siddiqui *et al*., 2011). We have approached this pharmacophores to suitably combine them through carbon–nitrogen–carbon bond formation for creating new hybrid molecules. Knoevenagel condensation of various heteroaromatic aldehydes with active methylene compounds like malononitrile, ethyl cyanoacetate, barbituric acid, meldrum's acid and dimedone proceeds smoothly with stirring under conventional heating (Jain *et al*., 2011). In addition, Knoevenagel condensation of aromatic aldehyde with organic compounds containing an active methylene group is a well-known process using a catalyst and organic solvent. In our efforts, devoted to green chemistry (Kisfaludy and Laszlo, 1987; Nerveux *et al*., 1991), herein we report a convenient, an environmentally friendly and a novel approach for the hybrid probes of aromatic amine and barbituric acid in aqueous medium with a very good yield. The significance of this protocol is to avoid the use of organic solvents and catalysis and ease of chemical transformation. Investigations of new leads screened for their tumour growth inhibitory activities against human cancer cell lines (*Breast adenocarcinoma cancer cell line* MCF-7, *Non-small cell lung cancer cell line* NCI-H460, *CNS cancer cell line* SF-268 and *fibroblast cancer cell line* WI-38) and antimicrobial activity against five bacterial strains (*S. pyogenes* MTCC 442 and *S. aureus* MTCC 96 as the gram positive, *E. coli* MTCC 443, *P. aeruginosa* MTCC 424 and *K. pneumoniae* MTCC 109 as the gram negative) and four fungal strains (*C. albicans* MTCC 227, *A. clavatus* MTCC 1323, *T. rubrum* MTCC 296 and *Penicillium wild strain*) and their results are discussed.

Results and discussion

Chemistry

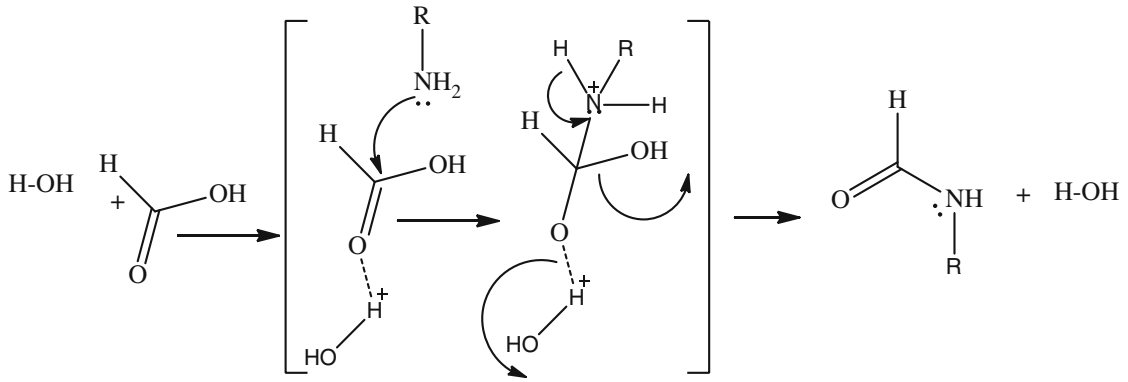
In this work, the synthesis of barbituric acid was carried out using Hantzch Reaction according to the literature procedure (Jacobson, 1937). In this study, as shown in Scheme 1, a series of 5-subtituted barbiturates **4** (**a**–**s**) has been synthesized with the concept of one pot three component system by condensation reactions of various aromatic amines **1** (**a**–**s**), formic acid **2** and barbituric acid **3** in aqueous medium with moderate to good yields (63–78 %). In accordance with the mechanism suggested in the literature (Schemes 2, 3) (Dhorajiya et al. 2014), the first step of this process may involve the *N-formylation* of aromatic amines with formic acid to form corresponding *N*-formamide derivatives. The second step, barbituric acid having active methylene functionality at 5th position undergoes knovenagel condensation with N-formamide derivatives yielded in the first step to form ***>C=CH–NH–*** of the adduct under the same conditions.

The final structures of all the new synthesized hybrid probes of aromatic amine and barbituric acid were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR, HMBC and Elemental Analysis. The important infrared spectral bands were recorded on Perkin Elmer—Spectrum RX-IFTIR using KBr discs. The characteristic peak at 1595.67 cm^{-1} of FTIR spectra of synthesized compounds is due to the presence of C=C group of aromatic ring, the peak at 1681.05 cm^{-1} confirming the presence of C=O group and peak at 3129.76 cm^{-1} attributed to N–H group of pyrimidine ring. The IR spectra of compounds **4** (**a**–**s**) revealed a characteristic bands at 2803.13 cm^{-1} confirming the presence of >C=CH–NH– group. ^{1}H NMR spectra were recorded on an Avance-II (Bruker) model using $CDCl_3$ as a solvent and TMS as internal standard with ^{1}H resonant frequency of 400 MHz. The ^{1}H NMR data of compounds revealed signals between 7.27 and 8.05 δ ppm for aromatic



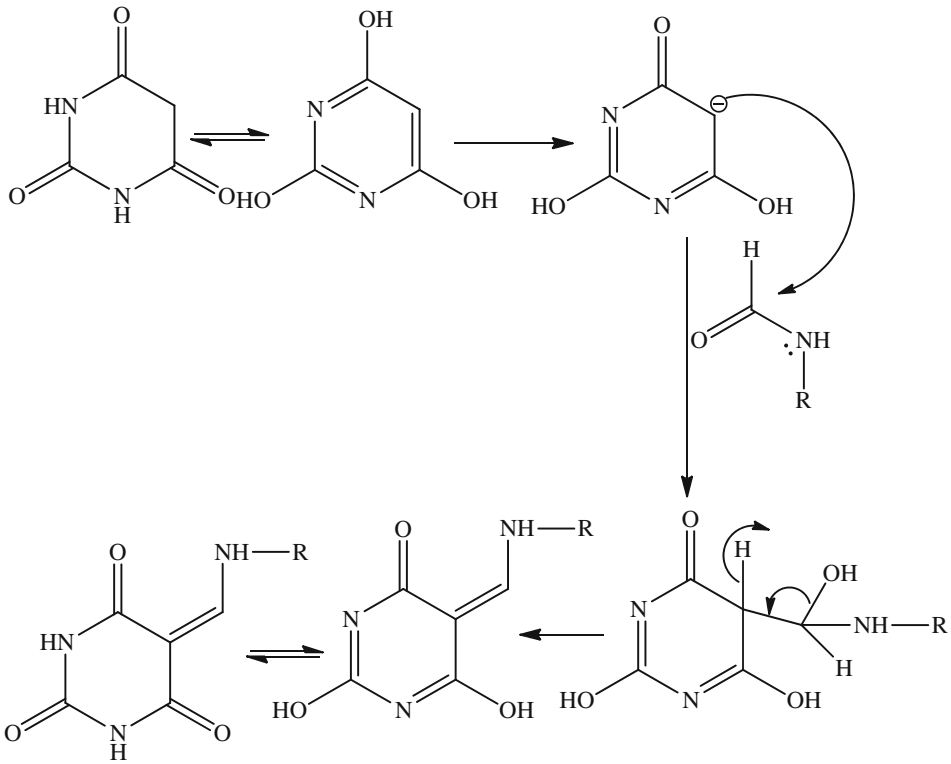
Reaction Conditions: Aromatic Amines (1 mmol), Formic acid (4 mmol), Barbituric acid (1 mmol) and Water (10ml).

Scheme 1 Synthesis for hybrid probes of aromatic amine and barbituric acid derivatives using water as green solvent



Scheme 2 Plausible reaction mechanism for *N*-formylation of aromatic amines with formic acid using water

Scheme 3 Plausible reaction mechanism for knoevenagel condensation in aqueous medium



protons of substituted phenyl ring. ^{1}H NMR data of compounds revealed signals at 8.62 δ ppm, confirming ***sp*** hybridization of carbon of >C=CH–NH–, and ***J*** value around 13.44 Hz confirms trans-geometry i.e. (**Z**)-configuration. ^{13}C NMR spectra were recorded on an Avance-II (Bruker) model using $CDCl_3$ as a solvent and TMS as internal standard with ^{1}H resonant frequency of 400 MHz. ^{13}C NMR spectra of compounds showed characteristic peak around 78.71, 94.21, 124.64, 131.70, 134.95, 139.95, 150.05, 151.23, 151.23, 164.91 and 167.60 δ ppm. U.V. Spectra were recorded on Maya pro 2000 (Ocean Optics USA) using DMSO as a solvent with 10^{-5} M solution. All synthesized probes **4** (**a**–**s**) showed the strong absorption bands (λ_{max}) in the range of 279.76–374.6 nm owing to the π–π* transition as well as the presence of exocyclic **CH** of the barbituric acid in the UV spectra (Silverstein and Webster, 1997).

Pharmacology

Structure activity relationship for anti-cancer activity assay

A close investigation of the effect of compounds on the growth of human tumour cell lines and a normal cell line Table 1. The anticancer activity assessed (Table 1) for analogues **4** (**a**–**s**) against four cell lines and demonstrated that some of the compounds revealed a good deal of activity against all the mentioned cell lines.

Table 1 Effect of compounds on the growth of human tumour cell lines and a normal cell line

Compounds	GI_{50} (μmol l^{-1})			
	MCF-7	NCI-H460	SF-268	WI 38
4a	2.6 ± 2.4	3.9 ± 0.8	1.8 ± 0.6	40.2 ± 10.2
4b	30.4 ± 10.2	20.1 ± 0.8	18.9 ± 6.8	44.1 ± 6.3
4c	32.1 ± 0.6	17.3 ± 1.4	22.3 ± 1.5	60.5 ± 22.6
4d	4.6 ± 1.2	3.6 ± 0.6	60.4 ± 1.8	1.3 ± 0.1
4e	20.6 ± 0.4	24.3 ± 0.8	32 ± 0.8	4.2 ± 1.8
4f	11.8 ± 0.6	14.5 ± 0.8	16.7 ± 1.6	>100
4g	38.0 ± 1.8	44.0 ± 0.8	20.5 ± 1.1	68.2 ± 12.9
4h	22.0 ± 0.2	30.6 ± 1.4	38.4 ± 0.6	30.1 ± 4.6
4i	33.7 ± 17.5	42.2 ± 12.8	54.0 ± 9.0	43.5 ± 8.2
4j	20.0 ± 0.6	22.0 ± 0.4	31.5 ± 8.0	58.2 ± 12.7
4k	0.9 ± 0.2	0.1 ± 0.02	0.3 ± 0.05	22.8 ± 8.0
4l	38.0 ± 1.8	44.0 ± 0.8	20.5 ± 1.1	10.3 ± 2.8
4m	0.01 ± 0.003	0.02 ± 0.001	0.01 ± 0.001	66.5 ± 12.7
4n	22.0 ± 0.2	30.6 ± 1.4	38.4 ± 0.6	44.3 ± 10.6
4o	22.7 ± 17.5	20.2 ± 12.8	33.0 ± 9.0	70.1 ± 22.3
4p	24.7 ± 11.5	22.2 ± 10.8	26.0 ± 8.0	40.7 ± 8.3
4q	0.03 ± 0.007	0.02 ± 0.008	0.01 ± 0.004	>100
4r	0.01 ± 0.002	0.02 ± 0.001	0.04 ± 0.008	>100
4s	32.0 ± 1.8	12.0 ± 0.8	14.5 ± 4.1	38.6 ± 4.7
Doxorubicin	0.04 ± 0.008	0.09 ± 0.007	0.09 ± 0.007	>100

GI_{50} mean value ± standard error of mean of 3 independent experiments performed in duplicate. MCF-7 breast adenocarcinoma, NCI-H460 non-small cell lung cancer, SF-268 (CNS cancer), WI 38-fibroblast cells

Subsequently, we may conclude from the above mention data the following structure activity relationship (SAR). The presence of the barbiturate moiety is necessary for the broad spectrum cytotoxic activity of the synthesized compounds towards different cell lines (MCF-7, NCI-H460, SF-268 and WI-38). Molecules **4m** having Electron Donating Group (**ED**) methyl group at *ortho* position showed (GI_{50} 0.01 ± 0.003 μM) against MCF-7 as the breast cancer cell line, (GI_{50} 0.02 ± 0.001 μM) NCI-H460 as the non-small cell lung cancer cell line and (GI_{50} 0.01 ± 0.001 μM) SF-268 as the CNS cancer cell line. Compounds **4q** having Electron Withdrawing Group (**EW**) functionality at *meta* position showed (GI_{50} 0.03–0.007 μM) against MCF-7 as the breast cancer cell line, (GI_{50} 0.02 ± 0.008 μM) NCI-H460 as the non-small cell lung cancer cell line and (GI_{50} 0.01 ± 0.004 μM) SF-268 as the CNS cancer cell line. Compounds **4r** having **EW** functionality at *para* position showed (GI_{50} 0.01–0.002 μM) against MCF-7 as the breast cancer cell line, (GI_{50} 0.02 ± 0.001 μM) NCI-H460 as the non-small cell lung cancer cell line and (GI_{50} 0.04 ± 0.008 μM) SF-268 as the CNS cancer cell line. For WI 38 cell lines, only three probes—**4f** having **EW** functionality carboxylic acid at *ortho* position and **4q** and **4r** having **EW** nitro functionality at meta and *para* position—exhibit (GI_{50} >100 μM). Moreover, all other probes showed highly potent efficacy towards WI-38 human fibroblast cell line. In addition, rest of the compounds indicated good to moderate cytotoxicity against MCF-7, NCI-H460 and SF-268 cell lines.

Structure activity relationship for anti-bacterial assay

A close investigation of the MIC values indicates that all the compounds exhibited a varied range of MIC (62.5–500 μg mL^{-1}) of anti-bacterial activity against all the mentioned bacterial strains. The MIC (Minimum inhibition Concentration in μ ml^{-1}) is given in Table 2.

For gram-positive bacteria, *S. pyogenes* compound **4e** consisting cyclohexyl ring shows closer activity (MIC = 62.5 μg mL^{-1}) as compared to standard drug ciprofloxacin (MIC = 50 μg mL^{-1}), while compound **4c** having **EW** group at *para* position, **4e** (cyclohexyl ring) and **4j** having **ED** chloride group at *ortho* position and **4q** having **EW** nitro functionality at *meta* position show activity (MIC = 100 μg mL^{-1}) against *S. aureus* and *S. pyogenes* as the pathogenic gram-positive bacterial strains which were two-fold higher than the standard drug ciprofloxacin (MIC = 50 μg mL^{-1}).

For gram-negative bacteria, *E. coli* as the bacterial strain compounds **4f** possessing **EW** carboxyl functionality at

Table 2 Effect of compounds on the growth of various bacterial strains

Compound	Minimum inhibitory concentration (MIC) expressed in μg ml^{-1}				
	Gram positive bacteria		Gram negative bacteria		
	S. pyogenes MTCC 442	*S. aureus* MTCC 96	*E. coli* MTCC 443	*P. aeruginosa* MTCC 424	*K. pneumoniae* MTCC 109
4a	500	500	250	250	100
4b	500	500	250	500	250
4c	100	125	250	500	200
4d	200	200	100	250	100
4e	62.5	100	250	500	250
4f	125	125	62.5	100	100
4g	200	125	100	125	125
4h	500	250	250	250	200
4i	250	200	200	100	500
4j	250	100	200	250	500
4k	500	500	125	250	125
4l	500	500	200	250	200
4m	200	250	250	250	500
4n	500	500	200	250	250
4o	200	250	250	500	250
4p	125	125	250	250	250
4q	100	100	200	250	200
4r	125	125	100	125	100
4s	250	250	125	125	125
Ciprofloxacin	50	100	25	25	25

MIC (μg mL^{-1}) minimum inhibitory concentration, that is, the lowest concentration of the compound to inhibit the growth of bacteria completely. *S. pyogenes*., *Streptococcus pyogenes*, *S. aureus*., *Staphylococcus aureus*, *E. coli*., *Escherichia coli*, *K. pneumonia*., *Klebsiella pneumonia*

para position shows closer activity (MIC = 62.5 μg/mL) as compared to standard drug ciprofloxacin (MIC = 25 μg/mL), while compounds **4d** having **ED** hydroxyl group at para position, **4f** with carboxylic acid as **EW** functionality at ortho position, **4 g** having **ED** methoxy group at *para* position, **4i** consisting **ED** Fluoro group at *para* position and **4r** having **EW** nitro group at *para* position show activity (MIC = 100 μg/mL) against *E. coli, P. aeruginosa* and *K. pneumoniae* as the pathogenic gram-negative bacterial strains which were four-fold higher than the standard drug ciprofloxacin (MIC = 25 μg/mL). Compounds **4 g** with **ED** methoxy group at *para* position, **4 k** having **ED** chloro group at *meta* position, **4r** with **EW** nitro group at *para* position and **4 s** without any substitution but exocyclic methyl group show (MIC = 125 μg/mL) against *E. coli, P. aeruginosa* and *K. pneumoniae* as the pathogenic gram-negative bacterial strains which were five-fold higher than the standard drug ciprofloxacin (MIC = 25 μg mL^{-1}). All the remaining compounds indicated good to moderate bacterial activity.

Structure activity relationship for anti-fungal assay

Anti-fungal activity was done by broth microdilution method. For assaying anti-fungal activity, *C. albicans*, *A. clavatus*, *T. rubrum* and *Penicillium wild strain* were re-cultured in DMSO. The MIC (Minimum Inhibition Concentration in μg mL^{-1}) is given in Table 3.

A close investigation of the MIC values indicates that all the compounds exhibited a varied range of MIC (250–1,000 μg mL^{-1}) of anti-fungal activity against all the tested fungal strains. The anti-fungal screening data of the compounds **4** (**a**–**s**) exhibit good to moderate activity. Moreover, against *T. rubrum* fungi compounds **4m** and **4n** having **ED** methyl substituent at *ortho* and *meta* position show an excellent activity (MIC = 250 μg mL^{-1}) as well as compound **4b** having naphthalene without any substitution, **4h** with **EW** sulphonic acid functional group at *para* position and **4o** having **ED** methyl group at *para* position show comparable activity with the reference griseofulvin as a standard (MIC = 500 μg mL^{-1}). Molecules **4g** with **EW** methoxy group at *para* position show

Table 3 Effect of compounds on the growth of various fungal strains

Compound	Minimum inhibitory concentration (MIC) expressed in μg ml^{-1}			
	C. albicans MTCC 227	*A. clavatus* MTCC 1323	*T. rubrum* MTCC 296	*Penicillium wild strain*
4a	1,000	>1,000	>1,000	1,000
4b	1,000	500	500	500
4c	500	1,000	1,000	>1,000
4d	1,000	>1,000	1,000	>1,000
4e	>1,000	>1,000	>1,000	500
4f	>1,000	>1,000	1,000	1,000
4g	250	500	1,000	200
4h	1,000	1,000	500	>1,000
4i	500	>1,000	1,000	1,000
4j	500	>1,000	>1,000	500
4k	250	>1,000	>1,000	>1,000
4l	1,000	500	>1,000	>1,000
4m	500	250	250	1,000
4n	500	250	250	500
4o	1,000	>1,000	500	500
4p	>1,000	1,000	1,000	>1,000
4q	500	>1,000	1,000	>1,000
4r	1,000	>1,000	>1,000	250
4s	500	>1,000	>1,000	1,000
Griseofulvin	100	100	500	100

MIC (μg mL^{-1}) minimum inhibitory concentration, that is, the lowest concentration of the compound to inhibit the growth of fungi completely. *C. albicans*., *Candida albicans*, *A. clavatus.*, *Aspergillus clavatus*, *T. rubrum., Trichophyton rubrum*

(MIC = 200 μg mL^{-1}) against *penicilium wild strain* fungi which were two-fold higher than the standard drug (MIC = 100 μg mL^{-1}). Compounds **4f** having **EW** carboxylic group at *ortho* position, **4k** with **ED** chloro group at *meta*, **4m** and **4n** with **ED** methyl substituent at *ortho* and *para* position, **4r** possessing **EW** nitro group at *para* position and **4s** without any substitution but exocyclic methyl group show (MIC = 250 μg mL^{-1}) against *C. albicans, A. clavatus* and *penicilum wild strain* fungi which were two and half fold higher than the standard drug griseofulvin (MIC = 100 μg mL^{-1}). Compounds **4c** with **EW** acetyl group at *para* position (R_1 = –H, R_2 = –H, R_3 = $–COCH_3$), **4d** with **ED** hydroxyl group at *para* position, **4f** with **EW** carboxylic group at *ortho* position, **4g** with **ED** methoxy group at *para* position **4i** with **ED** fluoro group at *para* position, **4p** and **4q** consist of **EW** nitro substituent at *ortho* and *meta* show activity (MIC = 1,000 μg mL^{-1}) which were two-fold higher than the reference to griseofulvin as a standard (MIC = 500 μg mL^{-1}) against *T. rubrum* fungi pathogen. All the remaining compounds indicated good to moderate fungal activity.

Experimental section

Materials and instrumentation

Chemicals and solvents were obtained from sigma aldrich and used as received throughout the investigation. Melting points were determined in open capillaries on a Veego electronic apparatus VMP-D (Veego Instrument Corporation, Mumbai, India) and are uncorrected. IR spectra (4,000–400 cm^{-1}) of synthesized compounds were recorded on a Perkin Elmer—Spectrum RX-IFTIR spectrophotometer using KBr pellets. Thin layer chromatography was performed on object glass slides (2 × 7.5 cm) coated with silica gel-G and spots were visualized under UV irradiation. ^1H NMR and ^{13}C NMR spectra were recorded on an Avance-II (Bruker) model using $CDCl_3$ as a solvent and TMS as internal standard with ^1H resonant frequency of 400 MHz and ^{13}C resonant frequency of 400 MHz. The ^1H NMR and ^{13}C NMR chemical shifts were reported as parts per million (ppm) downfield from TMS (Me_4Si). Mass Spectra were recorded on Micromass Q-Tof Micro. The splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; t, triplet; q, quartet; and m, multiplet. U.V. Spectra were recorded on Maya pro 2000 (Ocean Optics USA) using DMSO as a solvent with 10^{-5} M solution.

General procedure for synthesis of barbiturates of N-formylated aromatic amines

The barbiturate of *N*-formylated aromatic amine was synthesized by using different aromatic amines **1** (**a**–**s**) formic acid (2) and barbituric acid (3) via new green route of synthetic protocol. Initially, aromatic amines **1(a–s)** (1 mmol) and formic acid (4 mmol) were charged in a 3-neck flat bottom equipped with condenser, thermometer and stirrer. Deionized water was charge as a solvent in the flask. Reaction mass was continuously stirred for 2–3 h and temp was maintained 60 °C. Progress of reaction was monitored by TLC using hexane–ethylacetate (7:3) as the elluent system. After completion of reaction, reaction mass was cooled to room temperature and in situ barbituric acid (1 mmol) was charged. Reaction mass was further stirred at reflux temperature for 3–4 h. As the raw materials get converted into final products **4(a–s),** solid mass in the form of precipitates was formed. At the completion of reaction, precipitates were filtered, washed with water and methanol and dried (Dhorajiya and Dholakiya, 2013). All newly synthesized derivatives **4** (**a**–**s**) were characterized by using various spectroscopic techniques and screened for anti-cancer, anti-bacterial and anti-fungal activities.

Spectral characterization of synthesized compounds

*5-Phenylaminomethylene-pyrimidine-2,4,6-trione (**4a**)*

Light brown powder, Yield: 72 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1592.15 (C=C, aromatic), 1638.98 (NH), 2801.83 (C=CH–NH, exocyclic), 1678.97 (C=O), 3189.75 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.71 (1H, dd, N–H, $J = 3.25$), 7.21–7.95 (5H, m, –CH of aromatic ring), 8.68 (1H, dd, trans, exocyclic C–H, $J = 13.81$), 10.79 (1H, s, barbituric acid NH), 10.87 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 78.38 (C-5), 115.32 (C-13), 119.8(C-11, C15), 128.21 (C-12, C-14), 142.12 (C-10) 148.32 (C-7),151.23 (C-2), 164.91 (C-4, C-6); λ_{max}: 344.35 nm; €: 1.48×10^5 (L mol^{-1} cm^{-1}); M.W = 231.21; ESIMS: *m/z* 232.09 (M+1); Anal. calcd. For $C_{11}H_9N_3O_3$: C 57.14, H 3.92, N 18.17 (%). Found: C 57.19, H 3.89, N 18.19 (%).

*5-(Naphthalen-1-ylaminomethylene)-pyrimidine-2,4,6-trione (**4b**)*

Light brown powder, Yield: 69 %, M.P.185–188 °C, IR (KBr, cm^{-1}): 1593.27 (C=C, aromatic), 1638.73 (NH), 1679.67 (C=O), 2801.78 (C=CH–NH, exocyclic), 3190.26 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.73 (1H, dd, N–H, $J = 3.27$), 7.22–7.96 (7H, m, –CH of aromatic ring), 8.69 (1H, dd, trans, exocyclic C–H, $J = 13.82$), 10.81 (1H, s, barbituric acid NH), 10.89 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 78.91 (C-5), 109.32 (C-11), 119.21 (C-13), 121.64 (C-18), 124.9 (C-19),125.02 (C-17), 126.70 (C-15), 126.16 (C-16), 126.95 (C-12), 129.64 (C-15), 134.35 (C-14), 140.83 (C-10), 147.71 (C-7), 164.91 (C-4, C-6); *λmax*: 299 nm; €: 1.06×10^5 (L mol^{-1} cm^{-1}); M.W = 281.17; ESIMS: *m/z* 281.18 (M); Anal. calcd. For $C_{15}H_{11}N_3O_3$: C 64.05, H 3.94, N 14.94 (%). Found: C 64.09, H 3.91, N 14.89 (%).

*5-[(4-Acetyl-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4c**)*

Brown powder, Yield: 75 %, M.P. 211–215 °C, IR (KBr, cm^{-1}): 1595.36 (C=C, aromatic), 1643.02 (NH), 1727.78 (C=O, of Ar–$COCH_3$), 2803.13 (C=CH–NH, exocyclic), 1681.10 (C=O), 3193.08 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 2.55 (3H, s, –CH_3), 6.74 (1H, N–H, dd, $J = 3.24$), 7.24-8.11 (4H, m, –CH of aromatic ring), 8.67 (1H, dd, *trans*, exocyclic C–H, $J = 13.86$), 10.78 (1H, s, barbituric acid NH), 10.89 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 29.32 (C-17), 79.04 (C-5), 116.26 (C-12, C-14), 124.81 (C-13), 129.64 (C-11, C-15), 147.95 (C-7), 151.53 (C-2), 164.91 (C-4, C-6), 197.36 (C-17); λ_{max}: 355.53 nm; €: 1.30×10^5 (L mol^{-1} cm^{-1}); M.W = 273.24; ESIMS: *m/z* 275.12 (M+2); Anal. calcd. For $C_{13}H_{11}N_3O_4$: C 57.14, H 4.06, N 15.38 (%). Found: C 57.19, H 4.02, N 15.39 (%).

*5-[(4-Hydroxy-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4d**)*

Black powder, Yield: 67 %, M.P. >260 °C); IR (KBr, cm^{-1}): 1594.19 (C=C, aromatic), 1639.82 (NH), 2804.07 (C=CH–NH, exocyclic), 1680.76 (C=O), 3192.12 (N–H of pyrimidine ring), 3465.19 (*p*-Ar–OH); ^{1}H NMR (DMSO-d_6): δ ppm 5.0 (1H, s, –OH), 6.74 (1H, dd, N–H, $J = 3.28$), 7.35–8.11 (4H, m, –CH of aromatic ring), 8.67 (1H, dd, *trans*, exocyclic C–H, $J = 13.84$), 10.78 (1H, s, barbituric acid NH), 10.88 (1H, s, barbituric acid NH); ^{13}C NMR (DMSO-d_6): δ 80.29 (C-5), 116.72 (C-11, 15), 117.72 (C-12, C-14), 137.46 (C-10), 147.93 (C-7), 148.57 (C-13), 153.71 (C-5), 163.56 (C-4, C-6); λmax: 342.49 nm; €: 1.38×10^5 (L mol^{-1} cm^{-1}); M.W = 247.21; ESIMS: *m/z* 247.08 (M); Anal. calcd. For $C_{11}H_9N_3O_4$: C 53.44, H 3.67, N 17.00 (%). Found: C 53.39, H 3.72, N 16.99 (%).

*5-((Cyclohexylmino)methylene)pyrimidine2,4,6 (1H,3H,5H)-trione (**4e**)*

Cream powder, Yield: 65 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1593.28 (C=C, aromatic), 1640.47 (NH), 1682.35 (C=O), 2803.13 (C=CH–NH, exocyclic), 3190.64 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 1.43–2.57 (11H, m, –CH of cyclohexne ring, 6.72 (1H, dd, $J = 3.26$), 8.67 (1H, dd, *trans*, exocyclic C–H, $J = 13.85$), 10.79 (1H, s, barbituric acid NH), 10.86 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 23.3 (C-12, C14), 28.0 (C-13), 34.3 (C-11, C-15), 52.7 (C-10) 79.82 (C-5), 120.82 (C-11), 147.85(C-7), 149.97 (C-2), 165.09 (C-4, C-6); λ_{max}: 279. 76 nm; €: 1.05×10^5 (L mol^{-1} cm^{-1}); M.W = 237.26; ESIMS: *m/z* 238.25 (M+1); Anal. calcd. For $C_{11}H_{15}N_3O_3$: C 55. 69, H 3.30, N 6.37 (%). Found: C 55.72, H 3.25, N 17.74 (%).

*2-[(2,4,6-Trioxo-tetrahydro-pyrimidin-5-ylidenemethyl)-amino]benzoicacid (**4f**)*

Light brown powder, Yield: 70 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1595.67 (C=C, aromatic), 1642.17 (NH), 1681.05 (C=O), 1735.44 (C=O, *o*-Ar–COOH), 2803.13 (C=CH–NH, exocyclic), 3192.76 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.73 (1H, dd, N–H, $J = 3.28$), 7.27–8.05 (4H, m, –CH of aromatic ring), 8.62 (1H, dd, *trans*, exocyclic C–H, $J = 13.84$), 10.84 (1H, s, barbituric acid NH), 10.92 (1H, s, barbituric acid NH), 13.44 (1H, dd, –COOH, $J = 13.92$), ^{13}C NMR (DMSO-d_6): δ 78.71 (C-5), 94.21 (C-9), 124.64 (C-13), 131.70 (C-11), 134.95 (C-10),

139.95 (C-12), 150.05 (C-7), 151.23 (C-2), 164.91 (C-4, C-6), 167.60 (C-14); λ_{max}: 356.76 nm; €: 1.29×10^5 (L mol^{-1} cm^{-1}); M.W = 275.22; ESIMS: *m/z* 277.91 (M+2); Anal. calcd. For $C_{12}H_9N_3O_5$: C 52.37, H 3.30, N 15.27 (%). Found: C 52.39, H 3.29, N 15.26 (%).

*5-[(4-Methoxy-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4g**)*

Light brown powder, Yield: 71 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1596.73 (C=C, aromatic), 1642.17 (NH), 1683.27 (C=O), 2805.25 (C=CH–NH, exocyclic), 2827.35 (*p*-Ar–OCH_3), 3189.73 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 3.73 (3H, s, –OCH_3), 6.78 (1H, N–H, dd, $J = 3.24$), 7.31–8.12 (4H, m, –CH of aromatic ring), 8.69 (1H, dd, *trans*, exocyclic C–H, $J = 13.86$), 10.89 (1H, s, barbituric acid NH), 10.94 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 54.98 (C-16), 78.38 (C-5), 114.93 (C-11, C-15), 117.31 (C-12, C-14), 136.64 (C-13), 147.97 (C-10), 151.23 (C-2), 164.91 (C-4, C-6); λ_{max}: 342.49 nm; €: 1.31×10^5 (L mol^{-1} cm^{-1}); M.W = 261.23; ESIMS: *m/z* 261.15 (M); Anal. calcd. For $C_{12}H_{11}N_3O_4$: C 55.17, H 4.24, N 16.09 (%). Found: C 55.19, H 4.24, N 16.13(%).

*4-[(2,4,6-Trioxo-tetrahydro-pyrimidin-5-ylidenemethyl)-amino]-benzenesulfonic acid (**4h**)*

Cream powder, Yield: 63 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1174.26 (S=O of *p*-Ar–SO_3H), 1594.91 (C=C, aromatic), 1640.16 (NH), 1682.13 (C=O), 2804.13 (C=CH–NH, exocyclic), 3189.53 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 2.0 (1H, s, SO_3H), 6.77 (1H, dd, N–H, $J = 3.29$), 7.36–8.12 (4H, m, –CH of Aromatic ring), 8.67 (1H, dd, trans, exocyclic C–H, $J = 13.89$), 10.85 (1H, s, barbituric acid NH), 10.97 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 77.75 (C-5), 118.26 (C-11, 15), 128.96 (C-12, C-14), 140.14 (C-13), 147.24 (C-10), 151.02 (C-7), 153.73 (C-2), 163.94 (C-4, C-6); λ_{max}: 343.42 nm; €: 1.10×10^5 (L mol^{-1} cm^{-1}); M.W = 311.27; ESIMS: *m/z* 313.29 (M+2); Anal. calcd. For $C_{11}H_9N_3O_6S$: C 42.44, H 2.91, N 13.50 (%). Found: C 42.47, H 2.87, N 13.48 (%).

*5-[(4-Fluoro-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4i**)*

Cream powder, Yield: 74 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1117.35 (*p*-Ar–F), 1597.29 (C=C, aromatic), 1641.25 (NH), 1685.37 (C=O), 2803.59 (C=CH–NH, exocyclic), 3192.84 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.76 (1H, dd, N–H, $J = 3.26$), 7.34–8.19 (4H, m, –CH of Aromatic ring), 8.68 (1H, dd, *trans*, exocyclic C–H, $J = 13.85$), 10.84 (1H, s, barbituric acid NH), 10.94 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 77.61 (C-5), 116.32 (C-11, C-15), 117.96 (C-12, C-14), 135.52 (C-13), 140.64 (C-10), 147.92 (C-7), 150.26 (C-2), 164.79 (C-4, C-6); λ_{max}: 355. 02 nm; €: 1.42×10^5 (L mol^{-1} cm^{-1}); M.W = 249.20; ESIMS: *m/z* 250.06 (M+1); Anal. calcd. For $C_{11}H_8FN_3O_3$: C 53.02, H 3.24, N 16.86 (%). Found: C 53.05, H 3.27, N 16.83 (%).

*5-[(2-Chloro-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4j**)*

White powder, Yield: 72 %, M.P. >260 °C; IR (KBr, cm^{-1}): 735.24 (*o*-Ar–Cl), 1598.33 (C=C, aromatic), 1639.89 (NH), 1676.56 (C=O), 2802.76 (C=CH–NH, exocyclic), 3192.84 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.72 (1H, dd, N–H, $J = 3.27$), 7.21–8.05 (4H, m, –CH of Aromatic ring), 8.61 (1H, dd, *trans*, exocyclic C–H, $J = 13.79$), 10.77 (1H, s, barbituric acid NH), 10.91 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 78.38 (C-5), 117.72 (C-15), 120.27 (C-13), 125.64 (C-11), 127.74 (C-14), 129.76 (C-12), 143.95 (C-10), 149.64 (C-7), 151.54 (C-2), 164.69 (C-4, C-6); λ_{max}: 340.15 nm; €: 1.28×10^5 (L mol^{-1} cm^{-1}); M.W = 265.65; ESIMS: m/z 265.09 (M+1); Anal. calcd. For $C_{11}H_8ClN_3O_3$: C 49.73, H 3.04, N 15.82 (%). Found: C 49.70, H 3.07, N 15.79 (%).

*5-[(3-Chloro-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4k**)*

White powder, Yield: 69 %, M.P. >260 °C; IR (KBr, cm^{-1}): 756.49 (*m*-Ar–Cl), 1597.51 (C=C, aromatic), 1640.53 (NH), 1680.73 (C=O), 2801.98 (C=CH–NH, exocyclic), 3194.58 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 6.71 (1H, dd, N–H, $J = 3.29$), 7.23–8.07 (4H, m, –CH of aromatic ring), 8.64 (1H, dd, *trans*, exocyclic C–H, $J = 13.81$), 10.82 (1H, s, barbituric acid NH), 10.93 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 78.93 (C-5), 114.42 (C-11), 116.72 (C-15), 118.94 (C-13), 131.70 (C-12), 135.16 (C-14), 145.85 (C-10), 149.64 (C-7), 151.23 (C-2), 165.19 (C-4, C-6); λmax: 341.55 nm; €: 1.28×10^5 (L mol^{-1} cm^{-1}); M.W = 265.65; ESIMS: *m/z* 263.06 (M); Anal. calcd. For $C_{11}H_8ClN_3O_3$: C 49.73, H 3.04, N 15.82 (%). Found: C 49.71, H 3.06, N 15.84 (%).

*5-[(4-Chloro-phenyamino)-methylene]-pyrimidine-2,4,6-trione (**4l**)*

White powder, Yield: 67 %, M.P.244–247 °C; IR (KBr, cm^{-1}): 789. 16 (*p*-Ar–Cl), 1599.29 (C=C, aromatic), 1641.14 (NH), 1684.72 (C=O), 2804.11 (C=CH–NH, exocyclic), 3196.37 (N–H of pyrimidine ring); ^{1}H NMR

(DMSO-d_6): δ ppm 6.74 (1H, dd, N–H, $J = 3.31$), 7.27–8.09 (4H, m, –CH of aromatic ring), 8.64 (1H, dd, *trans*, exocyclic C–H, $J = 13.83$), 10.80 (1H, s, barbituric acid NH), 10.95 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 78.48 (C-5), 117.73 (C-11, C-15), 124.42 (C-13), 129.76 (C-12, C-14), 142.64 (C-10), 147.95 (C-7), 150.53 (C-2), 164.91 (C-4, C-6); λ_{max}: 355.02 nm; €: 1.39×10^5 (L mol^{-1} cm^{-1}); M.W = 265.65; ESIMS: *m/z* 267.04 (M+2); Anal. calcd. For $C_{11}H_8ClN_3O_3$: C 49.73, H 3.04, N 15.82 (%). Found: C 49.75, H 3.02, N 15.81 (%).

*5-[(o-Tolylamino)-methylene]-pyrimidine-2,4,6-trione (**4m**)*

White powder, Yield: 76 %, M.P. 252–255 °C; IR (KBr, cm^{-1}): 779 (*o*-Ar–CH_3), 1580 (C=C, aromatic), 1638 (NH), 2783 (C=CH–NH, exocyclic), 1727 (C=O), 3191 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 2.29 (3H, s, –CH_3), 6.69 (1H, dd, N–H, $J = 3.24$), 7.12–7.45 (4H, m, –CH of aromatic ring), 8.59 (1H, dd, *trans*, exocyclic C–H, $J = 13.78$), 10.85 (1H, s, barbituric acid NH), 10.92 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 23.78 (C-16), 79.64 (C-5), 116.32 (C-15), 118.72 (C-13), 126.6 -(C-14), 128.94 (C-11), 131.07 (C-12), 143.36 (C-10), 148.57 (C-7), 150.63 (C-2), 165.29 (C-4, C-6); λ_{max}: 341.55 nm; €: 1.39×10^5 (L mol^{-1} cm^{-1}); M.W = 245.23; ESIMS: *m/z* 247.13 (M+2); Anal. calcd. For $C_{12}H_{11}N_3O_3$: C 58.77, H 4.52, N 17.13 (%). Found: C 58.79, H 4.49, N 17.14 (%).

*5-[(m-Tolylamino)-methylene]-pyrimidine-2,4,6-trione (**4n**)*

White powder, Yield: 65 %, M.P. >260 °C; IR (KBr, cm^{-1}): 782 (*m*-Ar–CH_3), 1587 (C=C, aromatic), 1643 (NH), 1735 (C=O), 2795 (C=CH–NH, exocyclic), 3241 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 2.35 (3H, s, –CH_3), 6.72 (1H, dd, N–H, $J = 3.26$), 7.04-7.32 (4H, m, –CH of aromatic ring), 8.54 (1H, dd, *trans*, exocyclic C–H, $J = 13.82$), 10.83 (1H, s, barbituric acid NH), 10.98 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 20.89 (C-16), 78.81 (C-5), 115.25 (C-15), 118.41 (C-11), 126.49 (C-13), 129.49(C-14), 138.18 (C-12), 139.44 (C-10), 150.62 (C-7), 150.94 (C-2), 163.46 (C-4), 166.22 (C-6); λ_{max}: 344.82 nm; €: 1.40×10^5 (L mol^{-1} cm^{-1}); M.W = 245.23; ESIMS: *m/z* 246.17 (M +1); Anal. calcd. For $C_{12}H_{11}N_3O_3$; C 58.77, H 4.52, N 17.13 (%). Found: C 58.75, H 4.51, N 17.15 (%).

*5-[(p-Tolylamino)-methylene]-pyrimidine-2,4,6-trione (**4o**)*

White powder, Yield: 69 %, M.P. >260 °C; IR (KBr, cm^{-1}): 795 (*p*-Ar–CH_3), 1595 (C=C, aromatic), 1648 (NH), 1739 (C=O), 2807 (C= CH–NH, exocyclic), 3196 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 2.39 (3H, s, –CH_3), 6.78 (1H, dd, N–H, $J = 3.29$), 6.98–7.36 (4H, m, –CH of aromatic ring), 8.74 (1H, dd, *trans*, exocyclic C–H, $J = 13.87$), 10.81 (1H, s, barbituric acid NH), 10.94 (1H, s, barbituric acid NH),^{13}C NMR (DMSO-d_6): δ 22.34 (C-14), 79.12 (C-5), 116.32 (C-11,C-15), 128.5(C-13), 129.49 (C-12, C-14), 141.64 (C-10), 149.47 (C-7), 150.63 (C-2), 165.79 (C-4, C-6); λ_{max}: 343.89 nm; €: 1.40×10^5 (L mol^{-1} cm^{-1}); M.W = 245.23; ESIMS: *m/z* 245.51 (M); Anal. calcd. For $C_{12}H_{11}N_3O_3$: C 58.77, H 4.52, N 17.13 (%). Found: C 58.76, H 3.50, N 17.17 (%).

*5-[(2-Nitro-phenylamino)-methylene]-pyrimidine-2,4,6-trione (**4p**)*

Yellow powder, Yield: 71 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1343.46 (*o*-Ar–NO_2), 1592.94 (C=C, aromatic), 1640.39 (NH), 1679.97 (C=O), 2804.35 (C=CH–NH, exocyclic), 3187.76 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ 6.78 (1H, dd, N–H, $J = 3.29$), 7.32–7.93 (4H, m, –CH of aromatic ring), 8.67 (1H, dd, *trans*, exocyclic C–H, $J = 13.84$), 10.81 (1H, s, barbituric acid NH), 10.95 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 80.87 (C-5), 117.39 (C-11), 119.94 (C-13), 121.96 (C-14), 135.70 (C-15), 136.76 (C-12), 141.95 (C-10), 147.37 (C-7), 151.23 (C-2), 164.59 (C-4, C-6); λmax: 324.28 nm; €: 1.17×10^5 (L mol^{-1} cm^{-1}); M.W = 276.21; ESIMS: *m/z* 277.09 (M+1); Anal. calcd. For $C_{11}H_8N_4O_5$; C 47.83, H 2.92, N 20.28 (%). Found: C 47.84, H 2.89, N 20.29 (%).

*5-[(3-Nitro-phenylamino)-methylene]-pyrimidine-2,4,6-trione (**4q**)*

Light yellow powder, Yield: 75 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1359.86 (*m*-Ar–NO_2), 1595.82 (C=C, aromatic), 1681.63 (C=O), 1641.67 (NH), 2806.46 (C=CH–NH, exocyclic), 3190.87 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ 6.75 (1H, dd, N–H, $J = 3.33$), 7.35-7.95 (4H, m, –CH of aromatic ring), 8.70 (1H, dd, *trans*, exocyclic C–H, $J = 13.87$), 10.83 (1H, s, barbituric acid NH), 10.97 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 80.61 (C-5), 109.32 (C-15), 111.94 (C-11), 123.64 (C-11), 135.5 (C-12), 145.67 (C-10), 146.70 (C-10), 147.04 (C-7), 149.26 (C-14), 151.23 (C-2), 164.91 (C-4, C-6); λ_{max}: 332.22 nm; €: 1.20×10^5 (L mol^{-1} cm^{-1}); M.W = 276.21; ESIMS: *m/z* 276.23 (M); Anal. calcd. For $C_{11}H_8N_4O_5$: C 47.83, H 2.92, N 20.28 (%). Found: C 47.80, H 2.93, N 20.31 (%).

*5-[(4-Nitro-phenylamino)-methylene]-pyrimidine-2,4,6-trione (**4r**)*

Greenish yellow powder, Yield: 72 %, M.P. 165–168 °C, IR (KBr, cm^{-1}): 1376.17 (*p*-Ar–NO_2), 1598.06 (C=C,

aromatic), 1642.81 (NH), 1683.26 (C=O), 2808.91 (C=CH–NH, exocyclic), 3193.48 (N–H of pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ 6.72 (1H, dd, N–H, $J = 3.37$), 7.39–7.97 (4H, m, –CH of aromatic ring), 8.67 (1H, dd, *trans*, exocyclic C–H, $J = 13.89$), 10.85 (1H, s, barbituric acid NH), 10.99 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 80.34 (C-5), 117.73 (C-11, C-15), 122.13 (C-12, C-14), 138.47 (C-13), 147.79 (C-7), 149.96 (C-10), 152.23 (C-2), 164.91 (C-4, C-6); λmax: 374. 6 nm; €: 1.35×10^5 (L mol^{-1} cm^{-1}); M.W = 276.21; ESIMS: *m/z* 278.15 (M+2); Anal. calcd. For $C_{11}H_8N_4O_5$: C 47.83, H 2.92, N 20.28 (%). Found: C 47.86, H 2.91, N 20.29 (%).

*5-[(Benzylamino)-methylene]-pyrimidine-2,4,6-trione (**4s**)*

Cream powder, Yield: 78 %, M.P. >260 °C; IR (KBr, cm^{-1}): 1593.91 (C=C<, aromatic), 1640.27 (NH), 1681.09 (C=O), 2807.57 (C=CH–NH, exocyclic), 3192.76 (*N*-Hof pyrimidine ring); ^{1}H NMR (DMSO-d_6): δ ppm 3.91 (2H, s, –CH_2), 6.76 (1H, dd, N–H, $J = 3.27$), 7.22–8.02 (5H, m, –CH of aromatic ring), 8.63 (1H, dd, *trans*, exocyclic C–H, $J = 13.85$), 10.80 (1H, s, barbituric acid NH), 10.94 (1H, s, barbituric acid NH), ^{13}C NMR (DMSO-d_6): δ 52.17 (C-9), 81.63 (C-5), 126.72 (C-13), 127.07 (C-11, C-15), 128.92 (C-12, C-14), 141.64 (C-10), 151.23 (C-2), 159.75 (C-7), 165.59 (C-4, C-6); λmax: 306.03 nm; €: 1.24×10^5 (L mol^{-1} cm^{-1}); M.W = 245.23; ESIMS: *m/z* 246.18 (M+1); Anal. calcd. For $C_{12}H_{11}N_3O_3$: C 58.77, H 4.52, N 17.13 (%). Found: C 58.79, H 4.51, N 17.19 (%).

Pharmacology

Anti-tumour activity tests

Reagents

Fetal bovine serum (FBS) and L-glutamine were from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and sulforhodamine B (SRB) were from Sigma Chemical Co. (Saint Louis, USA). Samples: Stock solutions of compounds **4** (**a**–**s**) was prepared in DMSO and kept at 20 °C. Appropriate dilutions of the compounds were freshly prepared just prior to the assays. Final concentrations of DMSO did not interfere with the cell growth.

Cell cultures

Four human tumour cell lines, MCF-7 (*breast adenocarcinoma*), NCI-H460 (*non-small cell lung cancer*) and SF-268 (*CNS cancer*) together with the WI-38 (*normal fibroblast cancer cell*) lines were used. MCF-7 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), and NCI-H460, SF-268 and WI 38 were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They grow as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5 % heat-inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U mL^{-1}, streptomycin 100 lg mL^{-1}), at 37 °C in a humidified atmosphere containing 5 % CO_2. Exponentially growing cells were obtained by plating 1.5×10^5 cells mL^{-1} for MCF-7 and SF-268 and 0.75×10^4 cells mL^{-1} for NCI-H460, followed by 24 h of incubation. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5 %) of DMSO used in each assay. The growth inhibition activity was assessed according to a slightly modified procedure performed at the National Cancer Institute, Developmental Therapeutics Program (Boyd and Paull, 1995). The cells were inoculated onto standard 96-well microtiter plates on day 0. The cell concentrations were adjusted according to the cell population doubling time (PDT): 1×10^4 mL^{-1} for MCF-7 2 and NCI-H460 (PDT = 20–24 h), 2×10^4 mL^{-1} for SF-268 cell lines (PDT = 33 h). Test agents were then added in five, ten-fold dilutions (10^{-8}–10^{-4} mol L^{-1}) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in working concentrations.

Tumour cell growth assay

The effects of cytotoxic activity of the synthesized compounds towards different cell lines (MCF-7, NCI-H460, SF-268 and WI-38) on the in vitro growth of human tumour cell lines were evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) in the '*In vitro,* Anticancer Drug Discovery Screen' that uses the protein-binding dye sulforhodamine B to assess cell growth (Boyd and Paull, 1995). Briefly, cells exponentially growing in 96-wellplates were exposed for 48 h to five serial concentrations of each compound, starting from a maximum concentration of 150 μM. Following this exposure period, adherent cells were fixed, washed and stained. The bound strain was solubilized and the absorbance was measured at 492 nm in a plate reader (Bio-Tek Instruments Inc., Power wave XS, Wincoski, USA). For each test compound and cell line, a dose–response curve was obtained and the growth inhibition of 50 % (GI_{50}), corresponding to the concentration of the compounds that inhibited 50 % of the net cell growth was calculated as described elsewhere (Skehan *et al.*, 1990). Doxorubicin was used as a positive control and tested in the same manner.

Anti-microbial assay

Paper disc diffusion technique (agar streak dilution method)

The synthesized hybrid probes of aromatic amine and barbituric acid derivatives were examined for anti-microbial activity against five bacteria (*S. aureus, S. pyogenes, E. coli, K. pneumoniae and P. aeruginosa*) and four fungal strains (*C. albicans, A. clavatus, T. rubrum and Penicillium wild strain*) species using paper disc diffusion technique (Gillespie, 1994). The Mueller–Hinton agar media were sterilized (autoclaved at 120 °C for 30 min), poured at uniform depth of 5 mm and allowed to solidify. The microbial suspension (10^5 CFU/mL) (0.5 McFarland Nephelometery Standards) was streaked over the surface of media using a sterile cotton swab to ensure even growth of the organisms. The tested compounds were dissolved in dimethyl sulfoxide to give solutions of 3.12–100 mg mL^{-1}. Sterile filter paper discs measuring 6.25 mm in diameter (Whatman no. 1 filter paper) previously soaked in a known concentration of the respective test compound in dimethyl sulfoxide were placed on the solidified nutrient agar medium that had been inoculated with the respective micro-organism and the plates were incubated for 24 h at 37 ± 1 °C. A control disc impregnated with an equivalent amount of dimethyl sulfoxide without any sample was also used and did not produce any inhibition. Ciprofloxacin and Griseofulvin (100 mg $disc^{-1}$) were used as control drugs for anti-bacterial and anti-fungal activity, respectively.

MIC of the compound was determined by agar streak dilution method (Hawkey and Lewis, 1994). A stock solution of the synthesized compound (100 mg mL^{-1}) in dimethyl sulfoxide was prepared and graded quantities of the test compounds were incorporated in a specified quantity of molten sterile agar, i.e. nutrient agar for evaluation of anti-bacterial and sabouraud dextrose agar for anti-fungal activity. The medium containing the test compound was poured into a Petri dish at a depth of 4–5 mm and allowed to solidify under aseptic conditions. A suspension of the respective micro-organism of approximately 10^5 CFU mL^{-1} was prepared and applied to plates with serially diluted compounds with concentrations in the range of 3.12–100 mg mL^{-1} in dimethyl sulfoxide and incubated at 37 ± 1 °C for 24 h (bacteria) or 48 h (fungi). The lowest concentration of the substance that prevents the development of visible growth is considered to be the MIC value.

Conclusion

In summary, we have successfully developed a new cascade assembly for hybrid probes on the basis of the biological significance of aromatic amines and barbituric acid through green route synthetic protocol. The significance of this protocol is to avoid the use of organic solvents and catalyst with the simple reaction conditions having economical and environmentally benign approach. All synthesized compounds were evaluated for their anti-cancer and anti-microbial activities. Out of set of 19 molecules, 3 molecules show significant anti-cancer activities against MCF-7, NCI-H460 and SF-268, whereas 16 molecules exhibit potent anti-tumour activity against WI-38 cell lines. From anti microbial activity, three molecules exhibit significant activity against both the gram-positive as well as gram-negative bacteria. On the other hand, two molecules having electron donating methyl functionality as substituent on phenyl ring also showed highly potent activity against *T. rubrum* fungi strain where as three molecules were found to exhibit equipotent against *T. rubrum* fungi strain with reference to griseofulvin as standard drugs. In future, this methodology would be applicable for alkylarylamines, diarylamines, alkylamines or dialkylamines for the further development of new biological entity.

Acknowledgments We are very much thankful to Gujarat Council on Science and Technology for financial support (Project No. Gujcost/MRP/202042/12-13/04) and Department of Applied Chemistry, SVNIT, Surat for providing laboratory facilities. We are also thankful to Mr. D. M. Thumar from Amoli Organic Pvt, Ltd, Baroda, Gujarat and Avtarsingh & Manishkumar from SAIF, Panjab *University*, Chandigarh, India for providing characterization facilities.

Conflict of interest All three authors contributed equally to this work.

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