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Original article

Regioselective synthesis of phenanthrenes and evaluation of their anti-oxidant based anti-inflammatory potential

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ABSTRACT

Regioselective synthesis of 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (**1**) and 9,10-Dihydro-2,7-Dimethoxyphenanthrene-1,5-diol (**2**) was achieved using regioselective methylation, Wittig reaction, intramolecular cyclization and hydrogenation as key steps. The synthesis was successfully completed in total of 15 steps with 3.3% overall yield in case of **1** and in total of 13 steps with 9.0% overall yield in case of **2**. All compounds (**1**–**4**) showed good antioxidant and anti-inflammatory activity in *in vitro* assays and these activities were found to be due the presence of phenolic hydroxyl groups.

1. Introduction

Tumor Necrosis Factor-alpha (TNF-α) is a cytokine and a primary mediator of inflammation. Various anti-inflammatory drugs, particularly biologics such as etanercept, infliximab and adalimumab, work on the principle of inhibiting TNF-α. Though these TNF-α inhibitors are highly specific, their long term use is associated with serious side effects including tuberculosis and cancer [1]. However, TNF-α still remains an attractive target for developing new anti-inflammatory drugs that could be administered orally, would have fewer side effects and would lead to cost effective therapy [2].

TNF-α activates many signaling cascades upon binding with its specific receptor on cell surface. Nuclear-Factor kappa B (NF-κB) is one of the prominent ones. NF-κB activation is facilitated by conditions associated with increased intracellular oxidative stress. Reactive oxygen species (ROS) generated during inflammation not only helps to kill pathogens but also acts as important signaling molecules by changing the intracellular redox balance. ROS are important mediators that initiate and propagate inflammatory responses by stimulating release of pro-inflammatory cytokine such as TNF-α. However, uncontrolled ROS generation leads to oxidative stress and become primary cause of a variety of inflammatory diseases [3]. Therefore attenuating ROS by an antioxidant is beneficial in the management of ROS mediated inflammatory diseases [4]. Several Indian traditional medicinal plants are considered useful for treating inflammation [5]. *Eulophia ochreata*, an orchid, is a traditional medicinal plant used in India for rejuvenating and aphrodisiac properties [6–8]. It is also shown to possess anti-bacterial [9,10], anti-inflammatory [11], anti-oxidant and anti-diabetic [12] potential. Based on 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging property, we had recently isolated 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (**1**) and 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**4**) from *E. ochreata* tuber extract [13]. The compound **1** had further been shown to inhibit inflammatory signaling mediated by toll-like receptor [14]. This highlights the radical scavenging and anti-inflammatory potential of compound **1**. The compound **1** had also been shown to possess anti-proliferative potential [15]. Compared to its regio-isomer 9,10-Dihydro-2,7-Dimethoxyphenanthrene-1,5-diol (eulophiol) (**2**) [16], very limited study has been done on 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (**1**), possibly due to scarcity of the sample. Flavanthridin (**3**) is a 9,10-dihydro-derivative of 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**4**). Although, the inhibitory effect of **3** on LPS-induced NO-production in RAW 264.7 cells has been demonstrated [17], evaluation of anti-oxidant potential of **3** has not been reported so far. In addition, the inhibitory effect of all of these compounds (**1**–**4**) (Fig. 1) on TNF-mediated inflammation has not yet been reported. Since, isolation of these compounds from *E. ochreata* in large scale is not feasible owing to its endangered status [18,19]; we first synthesized **1**, **2** and **3** chemically for structure–activity-relationship study and then evaluated these compounds for their anti-oxidant and anti-inflammatory potential.

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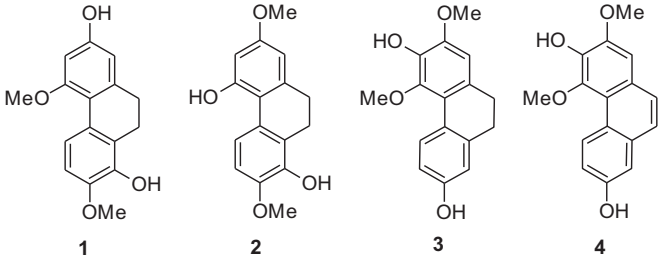


Fig. 1. Structure of 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (**1**), 9,10-Dihydro-2,7-Dimethoxyphenanthrene-1,5-diol (eulophiol) (**2**), Flavanthridin (**3**) and 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**4**).

2. Results and discussion

2.1. Synthesis

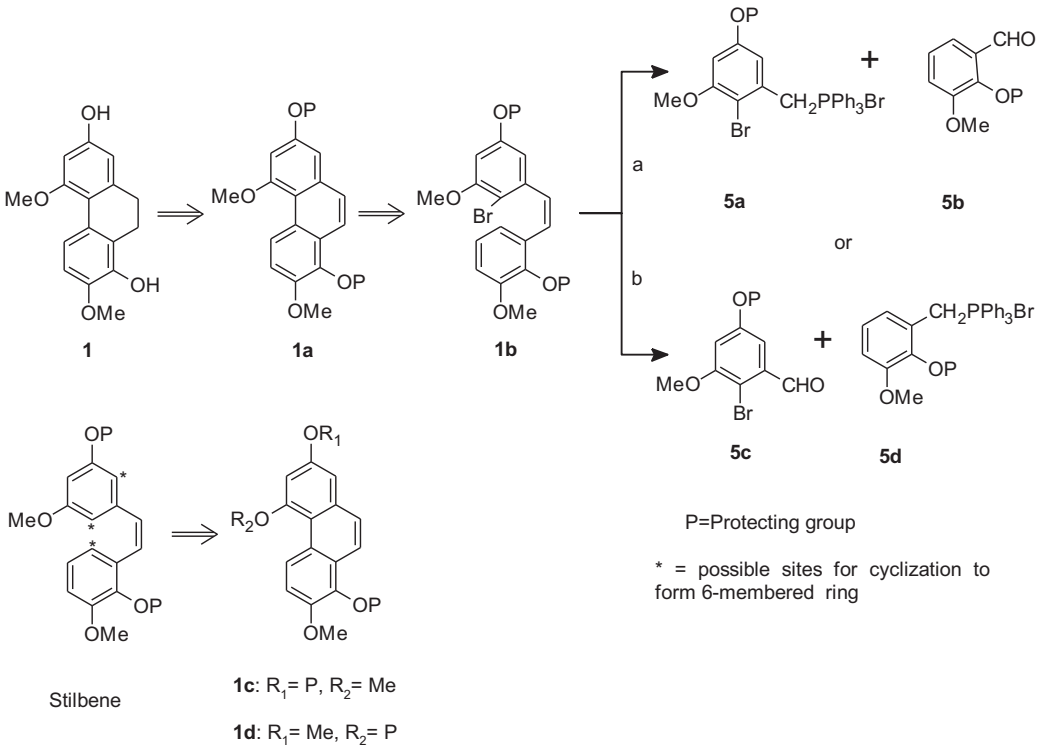
2.1.1. 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol (*1*)

Since synthesis of **1** has not been cited in the literature, we selected a reaction sequence comprising regioselective methylation, Wittig reaction, intramolecular radical cyclization and hydrogenation as the key steps due to commercial abundance of starting materials, *o*-Vanillin (2-hydroxy-3-methoxybenzaldehyde) (**6**) & orcinol (5-methylbenzene-1,3-diol) (**8**) (Scheme 1). As shown in Scheme 1, the intramolecular radical cyclization of stilbene would either give protected phenanthrene derivatives **1c** or **1d** which on further transformation would result in **1** or **2** respectively. Hence we employed bromo orcinol derivative (**5a** or **5c**) rather than bromo *o*-Vanillin derivative to achieve the regioselectivity in cyclization.

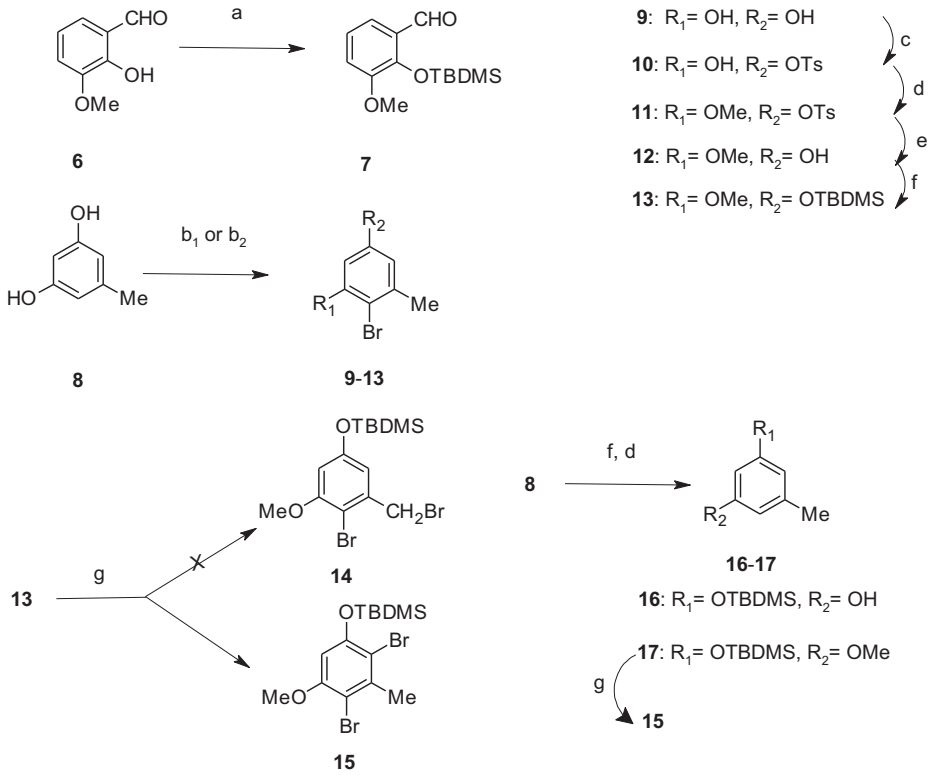
Synthesis of **1** commenced with silylation of *o*-Vanillin (**6**) to **7**. Its Wittig counterpart (**13**) was prepared from orcinol (**8**) *via* **9** [20,21] to **12** [22] and then silylation [23] with an overall yield of 39.2% from **8**. However, repeated attempts of converting **13** to **14** were unsuccessful which instead resulted in the formation of **15**. Alternative approach *via* **16** and **17** gave the same result. Though the presence of methoxyl group is known to cause bromination to occur at *para* position [24], formation of **15** was unexpected as high temperature and non-polar solvent were used in order to facilitate the side chain bromination *via* radical mechanism [25] (Scheme 2).

Having faced the problem of ring bromination on TBDMS protected phenol **13**, phenol **12** was acetylated (**18**), and then brominated (**19**). Since in Wittig reaction phosphonium salt interacts with a strong base (in this case n-butyl lithium), the presence of labile acetate group was undesirable. Therefore, we decided to replace it with TBDMS group, which is known to withstand Wittig conditions [26]. However deacetylation of **19** by various methods like methanolic HCl, CCl_4–HCl or refluxing methanolic HCl, HCl in acetone, NaOMe in MeOH, either failed or led solely to decomposition. As per Das et al. (2003) [27] phenolic acetates could be selectively removed using Amberlyst®-15 in MeOH at room temperature. However, it proved to be slow and generated an unidentified impurity in our case. Further transformation (**22**) followed by Wittig reaction with **7** generated a complex desilylated mixture (**23b** instead of **23a**) which on catalytic hydrogenation resulted in the formation of dihydrostilbene **24**. Owing to the perceived problem of removing acetate protecting group at benzyl bromide stage (**19**), alternative route b (Scheme 1) was trialed. The required component **27** was generated from **18** *via* tribromide **25** and aldehyde **26**. The other Wittig component **30** was derived from **7** *via* **28** and **29.** However the outcome of newly tried Wittig reaction did not change (Scheme 3).

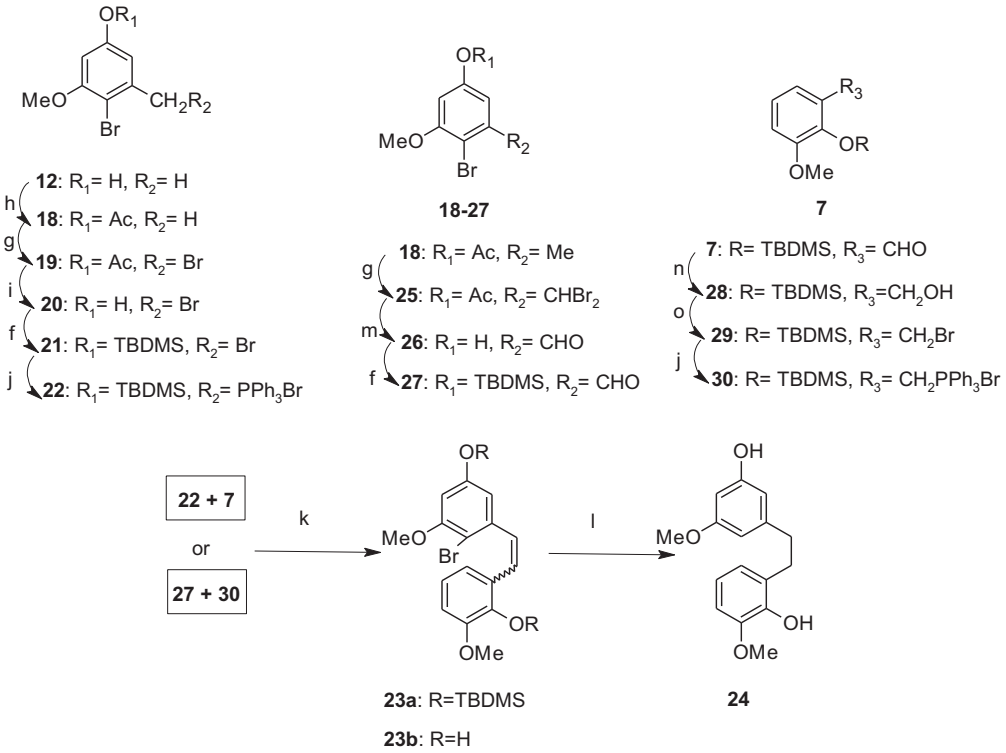
The presence of electron-withdrawing groups, Br in particular, at *para* position is known to expedite the deprotection of phenolic TBDMS ethers [28]. Therefore, the observed facile desilylation was suspected to be due to the presence of *para*-Br substituent (**22**, **27**). Also, it is known that the phenolic TBDMS ethers get easily deprotected by catalytic lithium hydroxide (LiOH) in DMF under milder conditions. According to Davies et al. (1992) [29], basic



Scheme 1. Retrosynthetic analysis of **1**.



Scheme 2. Synthesis of intermediates (**7**, **9**–**17**). Reagents and conditions: (a) TBDMSCl, DIPEA, THF, RT (87.9%); (b_1) $TBABr_3$, DCM:MeOH (3:2), 15–20 °C (70.1%); (b_2) Dioxane dibromide, diethyl ether, 15–20 °C (71.4%); (c) TsCl, K_2CO_3, acetone, reflux; (d) MeI, K_2CO_3, acetone, RT; (e) KOH, EtOH–H_2O, reflux (83.5%); (f) TBDMSCl, Immidazole, DCM; (g) NBS, CCl_4, benzoyl peroxide (cat.), reflux.



Scheme 3. Synthesis of Wittig components and their coupling by Wittig reaction. Reagents and conditions: (h) Ac_2O, pyr, RT; (i) Amberlyst®15, MeOH, RT, 24 h; (j) PPh_3, toluene, reflux, 4 h; (k) n-BuLi, −78 °C, THF; (l) H_2, 10%Pd(C), MeOH, RT, 24 h; (m) ammonium formate, EtOH:H_2O (1:1), reflux, 24 h; (n) $NaBH_4$, IPA, reflux, 2 h, (48.4%); (o) PBr_3, DCM, 0 °C, 45 min.

conditions favor cleavage of aryl silyl ether. Hence a small amount of DMF which was used to get a clear solution of compounds (**22**, **30**) in THF and formation of catalytic LiOH from n-BuLi in DMF could also be responsible for desilylation. Therefore benzyl protection was chosen because phenolic benzyl ether is known to be stable under these conditions [30]. Accordingly, **31** was synthesized from **26** with an overall 13% yield from **8**. For its Wittig counterpart, *o*-Vanillin (**6**) was benzylated (**32**), reduced (**33**), brominated (**34**) and then converted to phosphonium salt **35** with an overall 80.1% yield from **6**. It has been previously shown that the use of lithium methoxide (LiOMe) in DMF mainly led to Z-alkenes as the Wittig product [31–34]. Accordingly, Wittig reaction between **31** and **35** gave **36** which on AIBN–Bu_3SnH induced cyclization resulted in the desired phenanthrene **37.** The catalytic hydrogenation of **37** resulted in the formation of **1**. The outcome of the Wittig reaction is greatly influenced by number of factors which determine whether the Wittig reaction is under kinetic control or under thermodynamic control [35]. Electron donating groups make the ylide unstable and such unstable ylides react faster leading to kinetically controlled Z-alkenes. The presence of electron donating substituents in both the aldehyde and ylide appreciably enhances Z-selectivities of Wittig reaction [36–39]. In addition, when lithium salt in DMF is used, products are almost exclusively Z-alkenes [31,40]. The exclusivity of Z-**36** could hence be rationalized on the above mentioned grounds.

Though we reached to **1**, low overall yield and difficulty in final step purification demanded further development. The concerned impurity was generated due to suspected dehalogenation [37,41] of the stilbene **36** during radical cyclization. To circumvent this, the double bond of **36** was selectively reduced [42]. Further cyclization [43] of **38** was however unsuccessful. AIBN–Bu_3SnH induced cyclization of **38** resulted in the formation of **39a** along with the desired dihydrophenanthrene **39**. However, due to low yield of the desired dihydrophenanthrene **39** (6.94%) further work along these lines was abandoned and a hybrid methodology was adopted to reach effectively to **1**. Accordingly, **37** was first treated with *p*-$TsNHNH_2$, purified and then subjected to hydrogenation which yielded compound **1** with 97% purity (Scheme 4).

2.1.2. 9,10-Dihydro-2,7-dimethoxyphenanthrene-1,5-diol (eulophiol) (**2**)

Synthesis of eulophiol (**2**) has not been cited in the literature. Its synthesis was started with the preparation of Suzuki coupling partners: **41** from *o*-Vanillin (**6**) [44,45] and **42** from orcinol (**8**) [46,47] as per published protocols. Attention was next directed to the synthesis of arylboronic ester of either **41** or **42** by Miyaura protocol [48] because arylboronic acid synthesis using Grignard or lithium reagents requires the protection of functional groups (like CHO) which are sensitive to these reagents whereas Miyaura protocol tolerates various functional groups. Also, 2-Formyl group on arylboronic acid is known to accelerate the rate of hydrolytic deboronation [49]. However, our attempt to synthesize the boronate **41a** in DMF resulted in the formation of only a symmetrical biaryl **43c**. Alternative protocol [50] also resulted in the formation of symmetrical biaryls **43a** and **43c**. Formation of homodimers was presumably due to the Suzuki coupling of the boronate and its halide precursor under the reaction conditions [50]. Hence, an alternative reverse strategy was devised wherein boronate formation was attempted on **42**, followed by one-pot Suzuki-coupling with **41**. This yielded the desired cross coupled product (**43b**) along with the symmetrical biaryl (**43a**) (**43a**:**43b** 1:3.2).

Although we could not ascertain the exact cause of this deferential behavior of **41** and **42**, this could be due to benzyloxy group (OBn) as both the aldehydes differ from each other only in the position of OBn. Further, the formation of tetra-*ortho*-substituted symmetrical biaryl **43a** would be more difficult than tri-*ortho*-substituted cross-coupled biaryl **43b** due to steric hindrance. It was shown that the steric hindrance is not a major factor in aryl halides with one *ortho* group, however when ortho disubstituted Suzuki components (similar to **42a**) are used, the reaction gets adversely affected because of the steric hindrance during the transmetallation to the Palladium(II) complex [51]. Cross-coupled product (**43b**) on subsequent Wittig olefination gave divinyl **44**, which on ring-closing metathesis (**45**) followed by hydrogenation resulted in the formation of eulophiol (**2**) (Scheme 5). Low overall yield obtained in the above mentioned synthetic sequence demanded further development in the synthesis of eulophiol. At this point, we decided to use Wittig reaction since the required intermediates **35** (Scheme 4) and **42** (Scheme 5) were ready. Applying similar sequence we were able to synthesize eulophiol (**2**) with 93% purity (Scheme 6).

2.1.3. Semi-synthesis of flavanthridin (**3**)

Hydrogenation of **4** in EtOAc:EtOH (3:1) using Pd(C) followed by chromatographic purification resulted in the formation of **3** as white solid with 95% purity (Scheme 6).

2.2. Evaluation of biological functions

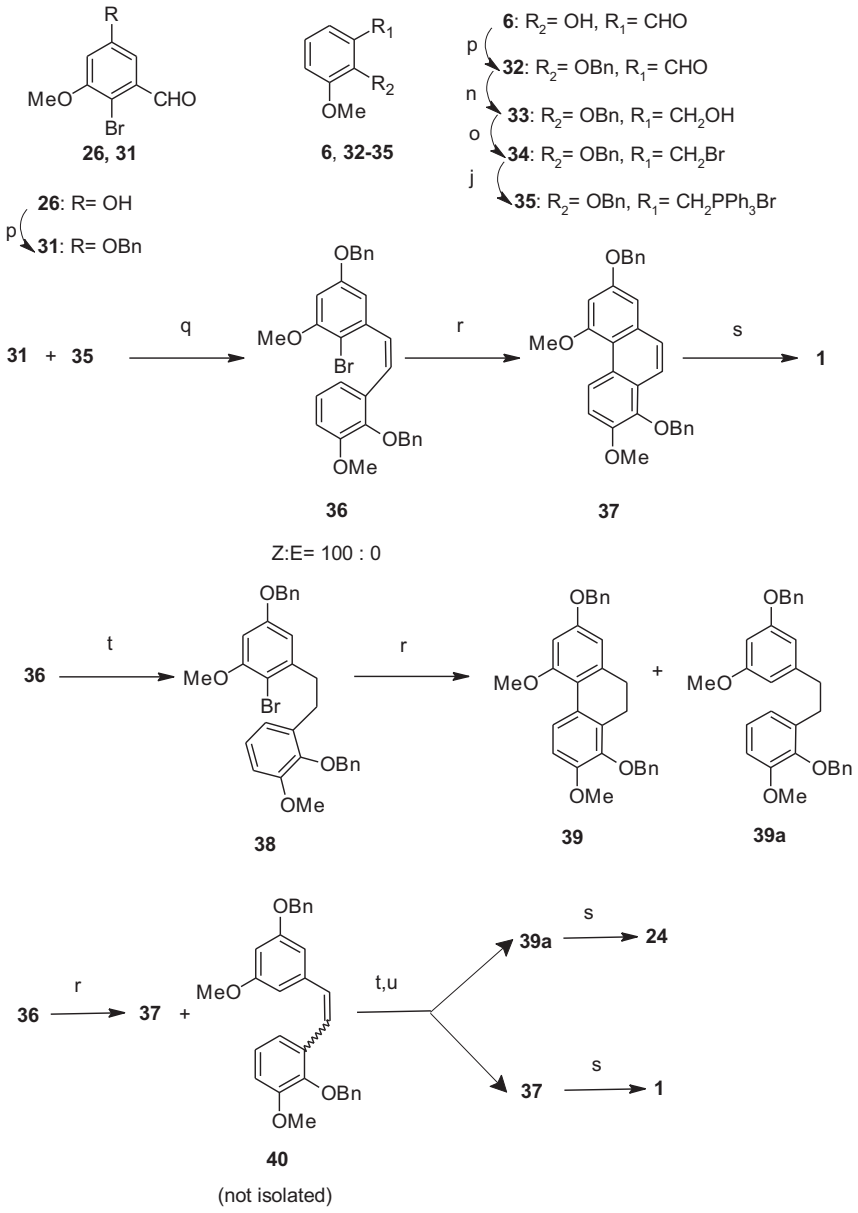
2.2.1. Free-radical scavenging activity of the compounds

Reactive oxygen species (ROS) have been implicated in a number of pro-inflammatory signal transduction cascades activated by cytokines such as IL-1β, TNF-α, and lipopolysaccharide. In this context, ROS have been considered second messengers. For example, ROS are generated in response to IL-1β, TNF-α, and lipopolysaccharide, and clearance of intercellular ROS can inhibit the ability of these ligands to activate downstream signals, including NF-κB [52]. Hence compounds with antioxidant activity would be beneficial in the management of inflammatory diseases. The radical scavenging potential of the compounds is generally assessed by using stable and colored radicals like DPPH, ABTS, and Galvinoxyl radicals. Both DPPH and Galvinoxyl radicals are commercially available whereas ABTS radical needs to be generated by oxidizing its neutral counterpart by potassium persulfate.

DPPH is a stable free radical with an absorption maximum at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species. The DPPH method is widely used for the determination of antiradical/antioxidant activity of purified phenolic compounds as well as natural plant extracts [53]. Hence we evaluated free radical scavenging potential of compounds **1**–**4** in DPPH assay. As shown in Table 1, compounds **3** and **4** exhibited significant DPPH scavenging activities which were higher than all the standards, such as Curcumin, Ascorbic acid, Catechin and BHT used in this assay whereas compounds **1** and **2** showed activities comparable to BHT. The order of reactivity of the compounds was **3** > **4** > **2** > **1** and that of the standards was curcumin > ascorbic acid > catechin > BHT.

Galvinoxyl is a stable phenoxyl radical and have characteristic absorbance maximum (λ_{max} = 428 nm) in methanolic solution. Unlike DPPH which is an N-centered radical, galvinoxyl is an O-centered radical and hence more closely related to physiologically acting oxygen radical [54]. When free radical scavenging potential of compounds **1**–**4** was tested in galvinoxyl assay, as shown in Table 1, the order of reactivity of the compounds was **3** > **4** > **1** > **2** and that of the standards was ascorbic acid > curcumin > catechin > BHT.

The bleaching of blue-green solution of $ABTS^{+\bullet}$ radical (λ_{max} = 734 nm) is a widely accepted method for evaluating the antiradical property of the compounds. $ABTS^{+\bullet}$ radical is more reactive than DPPH radical and in contrast to the reaction with DPPH



Scheme 4. Synthesis of **1** *via* radical cyclization of stilbene **36**. Reagents and conditions: (p) BnBr, K_2CO_3, DMF, 24 h, RT; (q) LiOMe, DMF, reflux, 1 h; (r) AIBN–Bu_3SnH, Toluene, reflux, 2 h; (s) H_2 (70 psi), 10% Pd(C), EtOAc–EtOH (3:1), 24 h; (t) *p*-$TsNHNH_2$ (5.0 equiv), NaOAc (2.0 equiv), EtOH, reflux, 2 h; (u) Si-gel column chromatography (Pet ether/EtOAc gradient) (66.6% **37** from **36**).

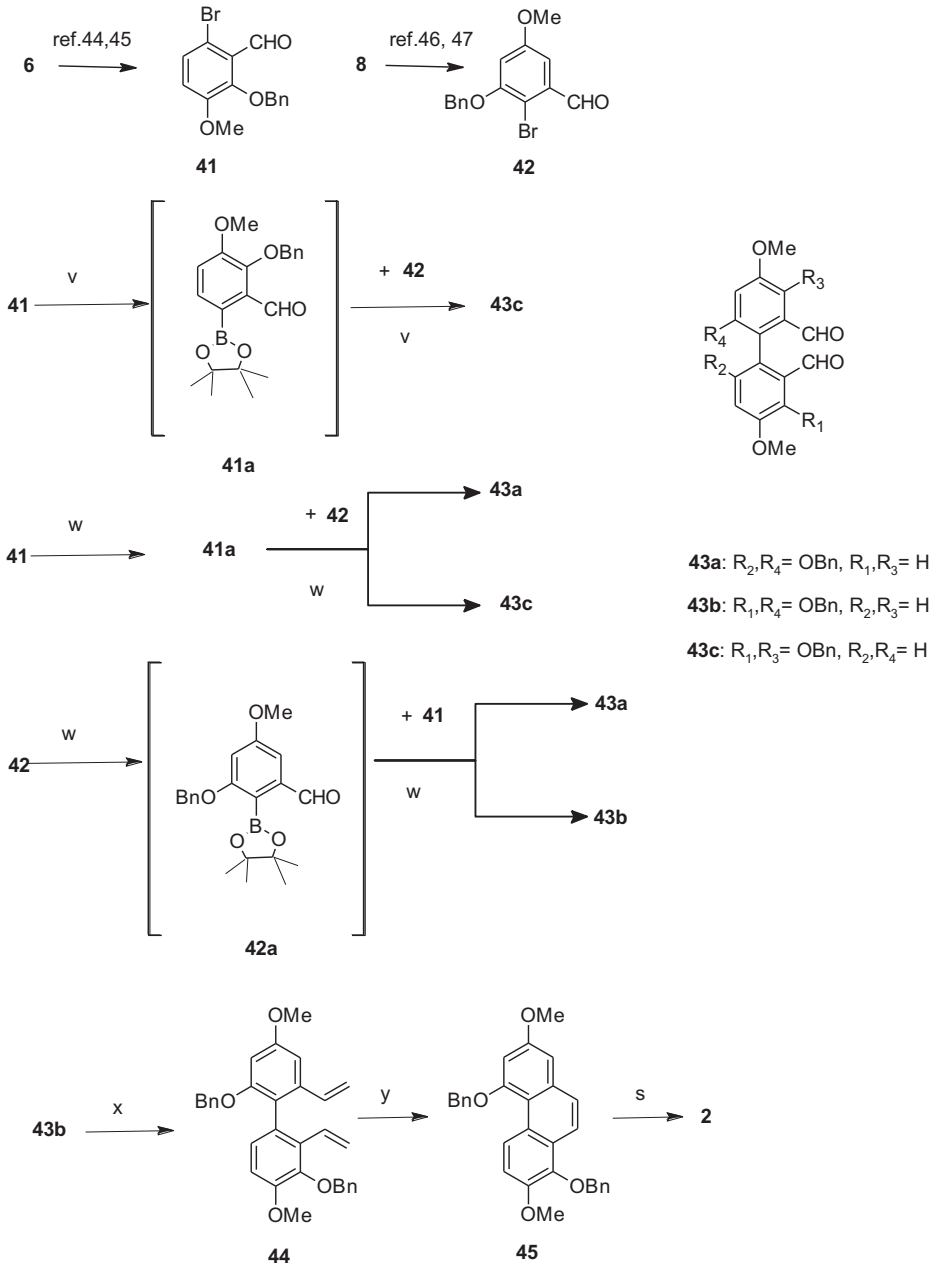
which is an H-atom transfer, its reaction involves a single electron transfer process [55]. When free radical scavenging potential of compounds **1**–**4** was tested in ABTS assay, as shown in Table 1, the order of reactivity of the compounds was **3** > **4** > **1** > **2** and that of the standards was catechin > curcumin > ascorbic acid > BHT.

2.2.2. Investigation into the structure–activity relationship

The compounds **1**, **2**, **3** and **4** possess two hydroxyl and two methoxy groups. Hence no significant differences were noted between IC_{50} values of **1** and **2** (both possess two OH). In addition to number of hydroxyl groups, the antiradical activity also depends on several other factors such as H-donating ability of the hydroxyl group, stability of phenoxyl radical, etc. [56]. The antiradical potency of the phenolic compounds is primarily governed by the bond dissociation energy of O–H bond. The presence of electron-donating group at *ortho*-position lowers the O–H bond dissociation enthalpy and increases the rate of H-atom transfer to radicals [57,58]. The unshared pair of electrons of *ortho*-OCH_3 in the p-orbital stabilizes the phenoxyl radical through electron delocalization and electron donation. Both **3** and **4** possessed two *ortho*-OCH_3. This might be the reason for their low IC_{50} values compared to that of **1** and **2**. This trend in radical scavenging efficiency of compounds **1**–**4** was maintained in all three methods utilized in this study. However, the differences in radical scavenging potential of two groups (compounds **3**–**4** versus compounds **1**–**2**) became more apparent in DPPH assay than in other two assays. Similar slow reactivity of DPPH with antioxidant is reported in the literature [53,59]. Both phenanthrene and dihydrophenanthrene backbone were found to be inactive (IC_{50} > 100) in all three assays due to absence of hydroxyl group.

2.2.3. Studies on TNF-α mediated biological effects

The release of TNF-α after stimulation of THP-1 cells with LPS is a valid model system to test novel compounds for potential



Scheme 5. Synthesis of **2** *via* Suzuki-coupling. Reagents and conditions: (v) Bis(pinacolato)diboron, PdOAc, KOAc, DMF, 80 °C; (w) Bis(pinacolato)diboron, $PdCl_2$(dppf), KOAc, DMSO, 80 °C, 2 h; then added other component, K_2CO_3, 80 °C, 2 h; (x) Ph_3PCH_3Br, n-BuLi, THF, −78 °C; (y) Grubbs 2nd generation Ru catalyst, DCM, 40 °C; (s) H_2 (70 psi), Pd/C, EtOAC (1.5% from **42**).

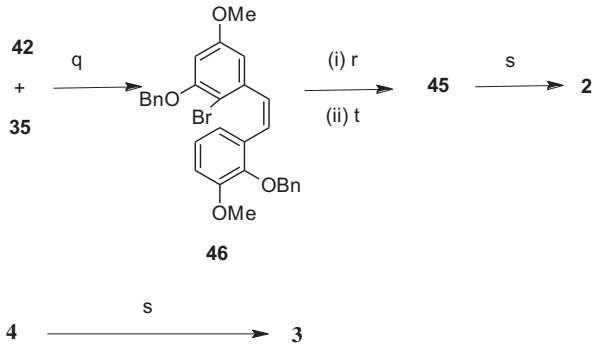
antiinflammatory effects [60]. Hence after LPS stimulation of THP-1 cells, TNF-α released was quantified by an Enzyme-linked immunosorbent assay (ELISA). Curcumin was treated as a positive control in the present study because it is known to inhibit NF-κB in different cell types including leukemia cells [61].

2.2.3.1. Inhibitory effects of compounds on LPS-induced TNF-α production in human THP-1 cells. THP-1 cells were exposed to varying concentrations of the compounds (1–100 μM) in a fresh serum-free medium in both the presence and absence of LPS. TNF-α was quantified by sandwich ELISA. As shown in Table 2, compounds **1**–**4** along with the positive control curcumin inhibited LPS-induced TNF-α secretion in THP-1 cells in a dose-dependent manner. On the other hand, their respective phenanthrene and dihydrophenanthrene backbone were inactive.

2.2.3.2. Cytotoxicity of isolated and synthetic compounds in THP-1 cells. The cytotoxicity of isolated and synthetic compounds was assayed to determine sample concentrations showing non-toxic effect on THP-1 cells.

As shown in Table 3, all four compounds **1**, **2**, **3**, and **4** along with their respective phenanthrene and dihydrophenanthrene backbone were found to be non-toxic to THP-1 cells, as against curcumin which showed toxicity at higher concentration (100 μM).

Curcumin is reported to be cytotoxic to THP-1 cells at higher concentration [62]. Therefore, in the present study, curcumin which exhibited the maximum inhibition of LPS-induced TNF-α was a result of reduced viability of THP-1 cells. As against this, the inhibitory effects of all four compounds **1**–**4** on LPS-induced TNF-α production in human THP-1 cells was not a result of reduced cell viability.



Scheme 6. Synthesis of **2** (*via* Wittig) and **3**.

3. Conclusion

The synthesis of 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol (**1**) was achieved in total of 15 steps with 3.3% overall yield and that of eulophiol (**2**) in total of 13 steps with 9.0% overall yield from orcinol (**8**). The results also corroborate the structure of isolated compound as 9,10-Dihydro-2,5-Dimethoxyphenanthrene-1,7-diol [13]. The synthesis of **2** reconfirmed the structure of eulophiol as 9,10-Dihydro-2,7-Dimethoxyphenanthrene-1,5-diol. A comparative antioxidant study based on three radical scavenging assays revealed the anti-inflammatory potential of compounds **1**–**4** and their potential in limiting ROS mediated inflammation. While their inhibitory potential demonstrated in THP-1 cells, non toxicity in THP-1 cell lines confirmed their activity profile. Inactivity of their corresponding phenanthrene and dihydrophenanthrene backbone in radical scavenging assays and THP-1 assay indicated that these activities are primarily due to phenolic hydroxyl groups. Our earlier research [15] toward this has demonstrated that isolated compound (**1**) down regulates expression of LPS-stimulated NF-κB-mediated, inflammatory cytokines *via* a Toll like receptor-mediated process. A similar mechanism could thus be responsible for anti-inflammatory activity shown by all other compounds **2**–**4** discussed in the present study. Successful chemical synthesis of these compounds will further reduce the dependence on the tuber extract of the endangered species (*E. ochreata*). These results may be helpful in future for the design of inhibitors of TNF mediated inflammation, and offer potential application in the discovery of anti-inflammatory drugs.

4. Experimental protocol

4.1. General methods

Melting points were determined using Lab India MR–VIS visual melting point apparatus and were uncorrected. Infrared spectra (IR) were recorded using KBr pellets on Perkin Elmer spectrum 100 series spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with Varian operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C with TMS as the internal reference (Sigma Aldrich). Mass spectra were obtained using Applied Biosystem MDS SCIEX 3200 QTRAP. Hydrogenation was carried out in rocker-shaker hydrogenator by Amar equipment. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F254 (0.20 mm layer, Merck) and the plates were examined under UV light at 254 nm and/355 nm. HPLC was performed on Dionex using analytical columns: column-1 (Waters Symmetry C18 5 μm 4.6 × 250 mm) with method 1 or column-2 (Kromasil C8 5 μm 4.6 × 250 mm) with method 2 (Table 4). Where ^{1}H NMR and HPLC were recorded on the diastereoisomeric mixtures, we had assumed that the more intense resonances correspond to the major isomer and that the less intense resonances were due to the minor isomer in accordance with the procedure adopted by Harrowven et al. (2006) [38]. Column chromatography was run on silica gel (60–120 mesh or 100–200 mesh) from ACME, India. All commercially available chemicals were used as received.

Table 1
Free-radical scavenging activity (IC_{50}) of the compounds.

Compound no.	IC_{50} (μg/mL)[b]		
	DPPH radical	Galvinoxyl radical	ABTS radical
1	32.5 ± 2.86	17.04 ± 1.31	15.62 ± 3.59
2	27.8 ± 4.97	17.97 ± 2.91	16.98 ± 1.92
3	10.28 ± 3.44	11.66 ± 2.76	11.72 ± 4.37
4	11.18 ± 3.79	11.79 ± 0.74	13.39 ± 5.85
Curcumin[a]	11.41 ± 2.36	17.03 ± 4.20	28.3 ± 7.16
Ascorbic acid[a]	15.12 ± 1.71	5.23 ± 1.14	29.17 ± 0.48
Catechin[a]	15.6 ± 1.17	18.03 ± 2.01	11.46 ± 0.18
BHT[a]	31.51 ± 2.84	43.7 ± 6.55	36.11 ± 1.94

[a] Positive control.
[b] Compounds tested in triplicate, data expressed as mean value ± SD of three independent experiments.

4.2. Cell culture and stimulation

Human acute monocytic leukemia cells (THP-1) were purchased from ATCC (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1% glutamine, 100 U/mL penicillin G and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was changed twice a week. THP-1 cells were pre-incubated with the compounds (1–100 μM) for 1 h, and then stimulated with the 250 ng/mL of lipopolysaccharide (LPS) (from *Escherichia coli* serotype O55:B5, Sigma) for 24 h.

4.3. Procedure for the synthesis of selected compounds

4.3.1. 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol (*1*)

To a solution of Phenanthrene **37** (0.8 g, 1.78 mmol) in EtOAc (30 mL) was added 10%Pd/C (360 mg), followed by EtOH (10 mL) and transferred the suspension to a rocker-shaker hydrogenator. Hydrogenator was pressurized to 70 psi with H_2 and shaken for 24 h. After HPLC monitoring, the suspension was passed through a celite bed and the filtrate was concentrated under reduced pressure. Crude product obtained was purified by silica gel column chromatography (Pet-ether: EtOAc 75:25) to get **1** (0.3 g, 62.5%) as white solid: mp 201–203 °C (reported [63] mp 202–203 °C); IR (KBr) ν_{max} 3445, 2948, 1602, 1498, 1481, 1442, 1276 cm^{-1}; ^{1}H NMR (400 MHz, CD_3COCD_3) δ 2.61 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 3.82 (s, 3H, OMe), 3.85 (s, 3H, OMe), 6.38 (d, 1H, J = 2.4 Hz, Ar-H), 6.45 (d, 1H, J = 2.4 Hz, Ar-H), 6.78 (d, 1H, J = 8.4 Hz, Ar-H), 7.19 (br s, 1H, D_2O exch., OH), 7.72 (d, 1H, J = 8.4 Hz, Ar-H), 8.24 (br s, 1H, D_2O exch., OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.69 (m, 2H, CH_2), 2.83 (m, 2H, CH_2), 3.86 (s, 3H, OMe), 3.91 (s, 3H, OMe), 5.67 (br s, 1H, D_2O exch., OH), 6.36 (m, 1H, Ar-H), 6.42 (m, 1H, Ar-H), 6.77 (d, 1H, J = 8.4 Hz, Ar-H), 7.78 (d, 1H, J = 8.4 Hz, Ar-H); ^{1}H NMR (400 MHz, CD_3SOCD_3) δ 2.61 (m, 2H), 3.29 (m, 2H), 3.76 (s, 3H, OMe), 3.79 (s, 3H, OMe), 6.29 (d, 1H, J = 2.4 Hz, Ar-H), 6.36 (d, 1H, J = 2.4 Hz, Ar-H), 6.77 (d, 1H, J = 8.8 Hz, Ar-H), 7.58 (d, 1H, J = 8.8 Hz, Ar-H), 8.35 (br s, 1H, D_2O exch., OH), 9.40 (br s, 1H, D_2O exch., OH); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 22.2 (CH_2), 29.7 (CH_2), 55.7 (OMe), 56.3 (OMe), 99.2, 108.2, 109.0, 116.3, 120.2, 124.7, 127.6, 141.6, 142.8, 145.0, 157.5, 159.1; EIMS: m/z 273 $(M + H)^+$; HPLC purity: 97.5% t_R: 22.8 min (column-1, method-1).

Similarly were synthesized other 9,10-Dihydrophenanthrene derivatives *Viz.* eulophiol (**2**) from (**45**) and flavanthridin (**3**) from

Table 2
Relative % TNF-α inhibition in THP-1 cells.[a,b]

Compound concentration	**1**	**2**	**3**	**4**	Phenanthrene	Dihydrophenanthrene	Curcumin
Cells + LPS	0	0	0	0	0	0	0
100 μM	83 ± 5.66	94 ± 0.93	64 ± 2.83	67.5 ± 3.54	8.5 ± 2.12	14.5 ± 6.36	102.5 ± 0.71
50 μM	70 ± 1.41	79 ± 4.24	47 ± 7.07	34 ± 0	6 ± 2.83	5 ± 4.24	95.5 ± 6.36
10 μM	31 ± 1.41	30 ± 0	17.5 ± 0.71	15.5 ± 2.12	0	0	38 ± 9.90
1 μM	19.5 ± 3.54	9 ± 7.78	8 ± 0	3.5 ± 0.71	0	0	1.5 ± 10.61

[a] Compounds tested in triplicate, data expressed as mean value ± SD of two independent experiments.
[b] TNF-α released in Cells + LPS taken as 100% TNF-α release (i.e. 0% TNF-α Inhibition).

3,7-dihydroxy-2,4-dimethoxyphenanthrene (**4**), physical constants and spectral data of **2** and **3** are summarized below.

*4.3.2. 9,10-Dihydro-2,7-dimethoxyphenanthrene-1,5-diol (**2**)*

White solid; Yield = 66.2%; mp 202–203 °C (reported [16] mp 202–203 °C); IR (KBr) ν_{max} 3446, 2964, 1616, 1436, 1465, 1355 cm^{-1}; ^1H NMR (400 MHz, CD_3COCD_3) δ 2.66 (m, 2H, CH_2), 2.77 (m, 2H, CH_2), 3.75 (s, 3H, OMe), 3.86 (s, 3H, OMe), 6.40–6.43 (m, 2H, Ar-H), 6.81 (d, 1H, J = 8.8 Hz, Ar-H), 7.27 (s, 1H, exch.D_2O., OH), 7.89 (d, 1H, J = 8.8 Hz, Ar-H), 8.45 (s, 1H, exch.D_2O, OH); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 21.6 (CH_2), 30.5 (CH_2), 54.7 (OMe), 55.6 (OMe), 100.9, 105.3, 108.4, 115.2, 119.5, 123.9, 127.1, 141.0, 142.3, 145.4, 155.6, 158.9; ^1H NMR (400 MHz, $CDCl_3$) δ 2.72 (m, 2H, CH_2), 2.81 (m, 2H, CH_2), 3.81 (s, 3H, OMe), 3.93 (s, 3H, OMe), 5.47 (s, 1H, exch.D_2O, OH), 5.76 (s, 1H, exch.D_2O, OH), 6.36–6.46 (m, 2H, Ar-H), 6.81 (d, 1H, J = 8.8 Hz, Ar-H), 7.51 (d, 1H, J = 8.8 Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.5 (CH_2), 30.2 (CH_2), 55.3 (OMe), 56.0 (OMe), 100.7, 106.5, 108.2, 114.7, 116.8, 124.4, 126.5, 141.3, 142.5, 145.0, 153.6, 158.9; EIMS m/z 273 $(M + H)^+$; HPLC purity: 93.15% t_R: 25.5 min (column-1, method-1).

*4.3.3. 3,7-Dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (**3**)*

White solid: mp 74–75 °C (reported [64] mp 75 °C); IR (KBr) ν_{max} 3207 (OH), 1610; ^1H NMR (400 MHz, $CDCl_3$) δ 2.71 (m, 4H), 3.70 (s, 3H, OMe), 3.91 (s, 3H, OMe), 5.26 (br s, 1H, D_2O exch., OH), 5.65 (br s, 1H, D_2O exch., OH), 6.57 (s, 1H, Ar-H), 6.74 (m, 2H, Ar-H), 8.16 (d, 1H, J = 8.8 Hz, Ar-H); EIMS: m/z 295 $(M + Na)^+$; HPLC purity: 95.9% t_R: 19.8 min (column-1, method-1).

4.4. Evaluation of the free-radical scavenging activity of the compounds

Free radical scavenging activities of the compounds were determined as per following published protocols [65–67]:

4.4.1. DPPH scavenging assay

The solution of each compound (5 μL) was added to 316 μM DPPH solution (95 μL) in 96 well plates. All the compounds were tested in final concentrations of 1, 5, 12.5, 25, 50 and 100 μg/mL respectively. The reaction mixture (100 μL) was mixed for 1 min and incubated at 37 °C for 30 min. The absorbance was measured at 517 nm on a microplate reader. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Radical scavenging activity } (\%) = \{1 - (A_1 - A_2)/A_0\} \times 100$$

where A_0 was the absorbance of the control (without test compound), A_1 was the absorbance in the presence of the test compound, and A_2 was the absorbance of sample blank (without radical).

4.4.2. Galvinoxyl radical scavenging assay

80 μM galvinoxyl methanolic solution (190 μL) was added to the methanolic solution of each compound (10 μL) (final concentration of compounds 1–100 μg/mL) in 96 well plates. The reaction mixture (200 μL) was mixed for 1 min and incubated at 37 °C for 20 min. The absorbance was measured at 428 nm on a microplate reader. The radical scavenging activity was calculated as described for DPPH assay.

4.4.3. $ABTS^{+\cdot}$ radical scavenging assay

The stock solution of ABTS (7 mM) was prepared by dissolving ABTS in water. $ABTS^{+\cdot}$ was generated by reacting ABTS stock solution with potassium persulfate (2.45 mM, final concentration) and allowing the mixture to stand in the dark for 16 h at room temperature. This stock solution was diluted with methanol to an absorbance of 0.70 (±0.02) at 734 nm. The diluted $ABTS^{+\cdot}$ solution (1.0 mL) was added to methanolic solution (20 μL) of compounds (final concentration of compounds 1–100 μg/mL) and absorbance was measured (734 nm) exactly after 1 min. The radical scavenging activity was calculated as described for DPPH assay.

4.5. Evaluation of inhibitory effects of compounds on LPS-induced TNF-α production

THP-1 cells were seeded in 96-well plates at a density of 2×10^5 cells/well. Cells were then exposed to varying concentrations of the compounds (1–100 μM) in serum-free medium and incubated for 1 h. To cells was then added LPS (250 ng/ml) dissolved in media, and incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. The cell supernatant (150 μL/well) was collected by centrifuging the

Table 3
Relative cell viability in THP-1 cells.[a,b]

Compound concentration	**1**	**2**	**3**	**4**	Phenanthrene	Dihydrophenanthrene	Curcumin
Untreated cells	1	1	1	1	1	1	1
100 μM	1.0 ± 0.05	1.1 ± 0.27	1.2 ± 0.04	1.0 ± 0.19	1.1 ± 0.16	1.2 ± 0.38	0.5 ± 0.03
50 μM	1.2 ± 0.21	1.2 ± 0.38	1.3 ± 0.08	1.1 ± 0.18	1.2 ± 0.27	1.3 ± 0.42	0.9 ± 0.00
10 μM	1.3 ± 0.27	1.4 ± 0.47	1.4 ± 0.16	1.3 ± 0.34	1.3 ± 0.31	1.5 ± 0.44	1.5 ± 0.21
1 μM	1.3 ± 0.29	1.5 ± 0.50	1.4 ± 0.02	1.4 ± 0.44	1.4 ± 0.27	1.6 ± 0.41	1.8 ± 0.08

[a] Compounds tested in triplicate, data expressed as mean value ± SD of two independent experiments.
[b] Untreated control cells = 1 (100% viable).

Table 4
Details of HPLC run.

Mobile phase	Water (%)	Acetonitrile (%)	Formic acid (%)
A	70	30	0.1
B	20	80	0.1
Time (min)		*%A*	*%B*
Method 1			
0		100	0
15		100	0
30		0	100
45		0	100
47		100	0
55		100	0
Method 2			
0		10	90
10		0	100
15		0	100
16		10	90
20		10	90

Flow = 1.0 mL/min, λ = 270 nm.

96 well plate at 500 × *g* for 5 min at 4 °C. TNF-α was then quantified by a standard sandwich ELISA. Sandwich ELISA was performed as per manufacturer (R&D system) protocol.

$$\text{Relative \% TNF-}\alpha\text{ Inhibition} = \times\left\{100-\frac{\text{TNF-}\alpha(\text{Cells}+\text{Compound}+\text{LPS})}{\text{TNF-}\alpha(\text{Cells}+\text{LPS})}\times 100\right\}$$

4.6. Evaluation of cytotoxicity

THP-1 cells were seeded in 96-well tissue culture plates at a density of 2×10^5 cells/well and then exposed to varying concentrations of the compounds (50 μL) in serum-free medium, incubated the plate for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. At the end of incubation, 10 μL of CCK-8 reagent was added to all the wells, incubated the plate for 2 h at 37 °C in a humidified 5% CO_2 atmosphere and read the plate (BioTek instrument; model Powerwave Xs with Gen 5 software) at 450 nm. The cell viability compared with the absorbance of the formazan in the controls without curcumin or the compounds was determined. The cell viability was reported relative to the absorbance of the untreated control (viability of control = 1).

$$\text{Relative Cell Viability} = \frac{\text{Absorbance}(\text{Cells}+\text{Compound})}{\text{Absorbance}(\text{Cells})}$$

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.06.016. These data include MOL files and InChiKeys of the most important compounds described in this article.

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