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Research paper

Synthesis and biological evaluation of salicylic acid conjugated isoxazoline analogues on immune cell proliferation and angiogenesis

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ABSTRACT

Mitogenicity is the ability of the natural or synthetic compounds to induce cell division or proliferation. A series of salicylic acid derivatives containing isoxazoline moiety (**8a-j**) were synthesized and their immunopharmacological activities targeting lymphocyte proliferation and angiogenesis were evaluated. The compounds **8a-j** mitogenicity were investigated on immunological cells that include human peripheral blood lymphocytes and murine splenocytes *in-vitro*. The results implicate that among the series of **8a-j,** compound **8e** showed a potent proliferative response on both human and murine lymphocytes. The proliferative index of the compound **8e** was comparable to the reference mitogen Con A and mitogenecity is due to increased secretion IL-2. *In -vivo* CAM and rat corneal angiogenesis assays were performed to assess the compound's effect on endothelial cell migration and proliferation which inferred that **8e** also induces the proliferation of endothelial cells. The study reports the synthetic immunostimulatory and pro-angiogenic activity of novel mitogen **8e** which could be translated into new drug in future.

1. Introduction

The immune system is an interactive network of lymphoid cells and proteins. The essential function of the immune system in host defense is best illustrated especially during its under activity which thereby results in host illness and vulnerability [1,2]. Therefore, immune activation of major immune cells, including T and B lymphocytes by immunostimulatory agents is likely to restore immune balance necessitating the discovery of such agents from synthetic or natural source [3,4]. Various such agents targeting immunoregulatory pathway were reported to stimulate lymphocytes through activating cytokines like interleukin-2 (IL-2) which potentially amplifies and expands lymphocyte growth [5]. Apart from this, a critical event in the immune response is the regulation of lymphocyte migration from the blood by vascular endothelial cells [6]. Various studies report that vascular endothelial cell proliferation is stimulated by the immunological cells, such as lymphocytes, thereby resulting in neovasculature [7]. Angiogenesis, which results from the complex cascade of endothelial cell proliferation plays an important role in pathological and physiological processes such as leucocytes recruitment, embryonic development, wound healing etc. As lymphocytes and endothelial cells have a strong association, the deregulated growth of either of those cells contributes to several complications that are accompanied with a notable state of immune suppression, impaired wound healing, ischemic diseases and myocardial infarction [8,9]. Hence, immunostimulatory compounds preferably with pro angiogenic efficacy would be novel therapeutic approaches to accelerate, reconstitute immunity deficiency and the neo vasculature to treat various angiogenic insufficiency disorders.

With traditional immunomodulatory drugs exhibiting limited efficacy and adverse side effects, the development of new, improved and specifically targeted immunotherapeutics is extremely important. However, immunomodulation, as a therapeutic need for the third millennium, remains in its infancy [10]. The design of hybrid drugs is one of the approaches to improve the efficiency of drugs to develop target specific action. Acetylsalicylic

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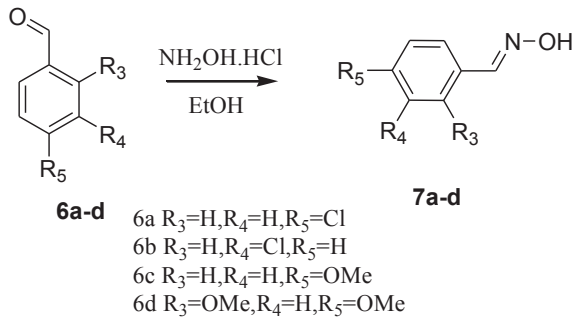
acid and its metabolite is well known for its pharmacological activities, like anti-inflammatory [11], anti-proliferative [3] etc. The anti-inflammatory activity of salicylates is due to its irreversible inhibition of cyclooxygenases (COX) which is a key enzyme in prostaglandin synthesis that are known to play a pivotal role in various inflammatory reactions. Beyond this there is also growing evidence indicating that some nonsteroidal anti-inflammatory drugs (NSAIDs) including acetylsalicylic acid may have additional immunomodulatory properties that amplify lymphocyte expansion [12,13]. On the other side isoxazoline analogues are attracting considerable attention due to their versatile applications in the field of medicine exhibiting varied pharmacological activities including immunomodulatory [3,14], vasodilation [4], insecticidal property [15,16], anticancer [17,18]. The immunological attributes of salicylic acid and isoxazoline derivatives envisaged us to synthesize a series of salicylic acid derivatives containing isoxazoline moiety (**8a-j**) with an intention to obtain a novel mitogenic and immunostimulatory agent. These salicylic acid-isoxazoline analogues were synthesized, via 1,3-dipolar cycloaddition reaction which is the versatile method for the synthesis of five membered heterocyclic compounds. The present study encompasses the immunopharmacological evaluation of synthesized salicylic acid conjugated 3, 5-diaryl isoxazoline analogues on immune cell proliferation and angiogenesis.

2. Results and discussion

2.1. Chemistry

The reaction of salicylic acid (**1**) with acetyl chloride and triethlyamine at 0–5 °C gave acetylsalicylic acid (**2**). The compound **2** was subjected to a Fries rearrangement in the presence of anhydrous aluminum chloride to obtain 5-acetyl-2-hydroxybenzoic acid (**3**). Further, condensation of compound **3** with benzaldehyde (**4a**) gave 5-(3-phenylacryloyl) 2-hydroxybenzoic acid (**5a**) as represented in Scheme 1. N-hydroxyl-1-(-3-chlorophenyl)-metanimine (**7a**) was obtained by the reported method [19] as represented in Scheme 2. Finally, the title compound 3(4-chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-chlorophenyl)4,5-dihydroisoxazoline (**8a**) was obtained by cycloaddition of nitrile oxide (which was prepared by oxidation of **7a** using chloramine–T) with compound **5a** as represented in Scheme 3.

All the synthesized compounds were characterized by IR, NMR and mass spectral studies. In the IR spectrum of compound **2,** the C=O band was observed at 1675 cm^{-1} and C–O at 1200 cm^{-1}. On the other hand, ^{1}HNMR spectrum of compound **2** showed signal at 2.8 ppm as a singlet for methyl group and the signal at 7.1–8.1 ppm as a multiplet for aromatic protons indicates the formation of



6a-d **7a-d**

6a R_3=H,R_4=H,R_5=Cl
6b R_3=H,R_4=Cl,R_5=H
6c R_3=H,R_4=H,R_5=OMe
6d R_3=OMe,R_4=H,R_5=OMe

Scheme 2. Synthesis of substituted N-hydroxy-1-phenylmethanimine (**7a-d**).

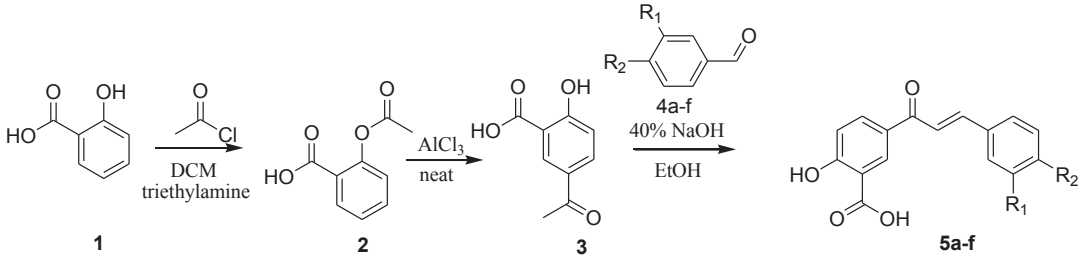
compound 2-acetoxybenzoic acid (**2**). Further, the appearance of a broad peak for phenolic OH at 3510-3610 cm^{-1} in the IR spectrum and a broad singlet at 10.6 ppm for OH group and decrease in the number of aromatic protons in ^{1}H NMR spectrum clearly indicates the formation of 5-acetyl-2-hydroxybenzoic acid **(3)**. Among the series of compounds **5a-f**, compound **5a** has been taken as a representative example to discuss spectral characterization, in the IR spectrum of compound **5a,** an absorption of C=C peak at 1635 cm^{-1} and increase in the number of aromatic protons with the appearance of two doublet at 7.3 ppm and 7.7 ppm indicating the formation of 5-(3-phenylacryloyl) 2-hydroxybenzoic acid (**5a**).

Similarly, among compounds **8a-j,** compound **8j** has been taken as a representative example to discuss spectral characterization in the IR spectrum of compound **8j** disappearance of C=C peak at 1635 cm^{-1}. On the other hand, in ^{1}H NMR spectrum of compound **8a,** showed a signal of proton at 3rd position appeared as a doublet at 6.7 ppm and the proton at 4th position exhibits peak at 5.8 ppm much upfield when compared to its precursor **5a** which indicates the cycloaddition to the double bond.

2.2. Biology

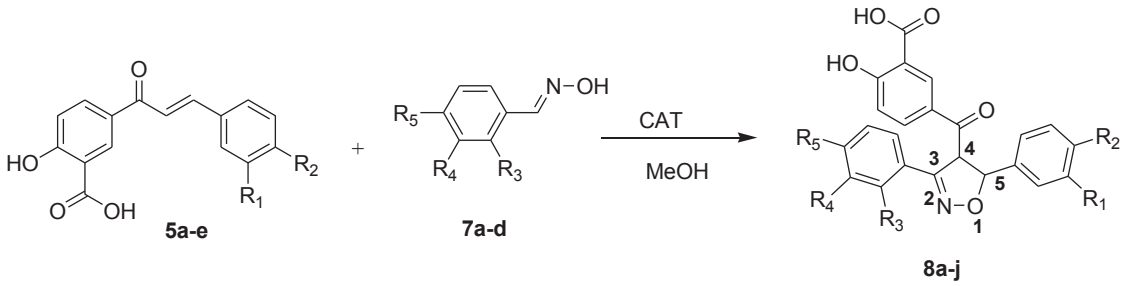
2.2.1. Structure Activity Relationship (SAR) and selection of *8e* as potent mitogen

The current investigation is focused to evaluate the biological implications of serially synthesized salicylic acid integrated dihydro-isoxazoline analogues (**8a-j**). As an approach, effects of **8a-j** on cellular proliferation were initially screened against Human Embryonic Kidney cells 293 (HEK-293) (Supplementary table 1). The outcome of the results inferred that among **8a-j** series, the compounds **8e** and **8g** showed a significant role in the activation and proliferation of HEK 293 than other compounds at 100 μM concentration and the results were tabulated in Supplementary table 1. Various reports have shown that isoxazoline derivatives



a R_1=H,R_2=H
b R_1=H,R_2=Br
c R_1=H,R_2=OH
d R_1=Cl,R_2=H
e R_1=H, R_2=OMe
f R_1=H,R_2=Cl

Scheme 1. Synthesis of 5-(3-phenylacryloyl) 2-hydroxybenzoic acid (**5a-f**).



8a R_1=H, R_2=Cl,R_3=H,R_4=H,R_5=Cl
8b R_1=H, R_2=Br,R_3=H,R_4=H,R_5=Cl
8c R_1=H, R_2=Br,R_3=H,R_4=Cl,R_5=H
8d R_1=H, R_2=OH,R_3=H,R_4=H,R_5=OMe
8e R_1=Cl, R_2=H, R_3=H,R_4=H,R_5=OMe
8f R_1=H, R_2=OH,R_3=H,R_4=Cl,R_5=H
8g R_1=Cl, R_2=H,R_3=H,R_4=Cl,R_5=H
8h R_1=H, R_2=OMe, R_3=H,R_4=H,R_5=Cl
8i R_1=H, R_2=OMe, R_3=H,R_4=Cl,R_5=H
8jR_1=H, R_2=H, R_3=OMe,R_4=H,R_5=OMe

Scheme 3. Synthesis of 3(4-chlorophenyl)-4(3-hydroxy-4-carboxy benzoyl)-5(4-chlorophenyl)4,5-dihydro dihydroisoxazoline (**8a-j**).

have the ability to modulate the innate immune response, indicating their potential as a source of new immunomodulatory agents. Though the isoxazoline derivatives were known to be as immunopotentiating compounds, it is of interest that isoxazoline derivatives with different substitution demonstrated differential activity in the proliferative ability. Some of the derivatives were known to potentially stimulate the immune cell proliferation, whereas some were known to exert immune suppressive activity by inhibiting the proliferative response [15]. In the current study, we have synthesized a series of compounds comprising salicylic acid coupled with isoxazoline moiety. Since salicylic acid and its derivatives are known to induce the mitogenic activity of lymphocytes [13,20] all the analogues (**8a-j**) were screened against human peripheral lymphocytes to study the effect on immune cell proliferation. A positive reference mitogen concanavalin A (Con A) [21] was employed in this study in the concentration range of 1–20 μg/mL. The compound **8e** bearing methoxy group at the para position of phenyl ring attached to 3rd position and chloro group at the meta position of phenyl ring attached to 5th position of heterocyclic ring showed an increase in the proliferative response of lymphocytes thereby demonstrating a highest proliferative index in concurrent with positive control Con A (Supplementary figure 1) in a concentration dependent manner. Compound **8e** has more than 1.25 fold proliferative index as compared to Con A which has 1.75. Some of the analogues (Supplementary figure 1) such as compound **8g** with both chloro groups at meta positions of the phenyl rings attached to the 3rd and 5th positions of the heterocyclic ring showed only a minimal stimulatory activity than the compound **8e**. The structural details of the compounds **8e** and **8g** revealed that both the compounds bearing chloro group at the meta position of phenyl ring attached to 5th position of heterocyclic ring, could be the reason for its increased proliferative response. Among the series of compounds **8a, 8b, 8c, 8f, 8h** and **8i** also comprising of the chloro group at different positions, none has exhibited the proliferative activity. Selective concentration (25, 50 & 100 μM) **8e** were depicted in Fig. 1A. Based on these results compound **8e** was considered as the most promising one and the same was chosen for the further investigations.

2.2.2. Compound *8e* proliferates murine lymphocytes in-vitro

To evaluate the efficacy of compound **8e** on other immune cells, murine splenocytes having a different subset population of lymphocytes was employed. The proliferative effect of **8e** and Con A on murine splenocytes showed 1.2 fold increases comparable to that of Con A, indicating that **8e** is mitogenic towards murine immune cells also (Fig. 2A and B). The index for the control (untreated cells) was taken as 1.0 and others are represented as fold increase or decrease over the control.

2.2.3. Compound *8e* influences interleukin secretions

The cytokines play an important role in immune system modulation by playing a vital role in cell proliferation, differentiation and maturation [22]. IL-2 is a potent activator of T-cell growth and differentiation and have an important role in immune therapy [23,24]. On other hand IL-12 and IL-23 are secreted by human dendritic cells and macrophages in response to exogenous and endogenous signals and associated with host defense and wound healing [25]. To check the possible expression of these cytokines we measured IL-2, IL-12 and IL-23 levels after compound **8e** treatment for 72 h. The compound **8e** stimulated the 4 fold increase in the level of IL-2 (Fig. 3). The control lymphocytes secreted 328 pg/mL of IL-2 where in treated compound **8e** was 1,456 pg/mL.The level of IL-12 and IL-23 were moderately altered, which was not so significant. This could be due to the absence of macrophages, dendritic cells in HPBL which contains mainly B lymphocytes, T lymphocytes and NK cells. Further investigations of these two cytokines are kept under study by specifically isolating macrophages and dendritic cells which secrete IL-12 and IL-23. Overall results infer that Compound **8e** proliferates lymphocytes through IL-2 expression and the role of IL-12/IL-23 are under investigation.

2.2.4. Compound *8e* inhibits COX-1 activity

Prostaglandin H2 synthase (cyclooxygenase or COX) is the rate limiting enzyme in the conversion of arachidonic acid (AA) to prostaglandins (PG). PG are important mediators in physiological processes and in the inflammatory response [26]. COX-1 is constitutively expressed by most cell types including T-Lymphocytes and it is thought to participate in PG synthesis during physiological processes. Many NSAID's such as acetyl salicylate is known to inhibit constitutively expressed COX-1 in T-Cells [27]. Studies have also reported that COX1 inhibition by acetyl salicyclate may account for some of their unwanted side effects [28,29]. To study the mode of action of compound **8e** we have measured the cellular COX-1 activity in Human PBL. The result inferred that compound **8e** inhibited COX-1 activity by 39%, which may be due to presence of acetylsalicylate moiety (Fig. 4). The detailed study on the effect of **8e** on other COX expression is kept under investigation for further validation of the molecule.

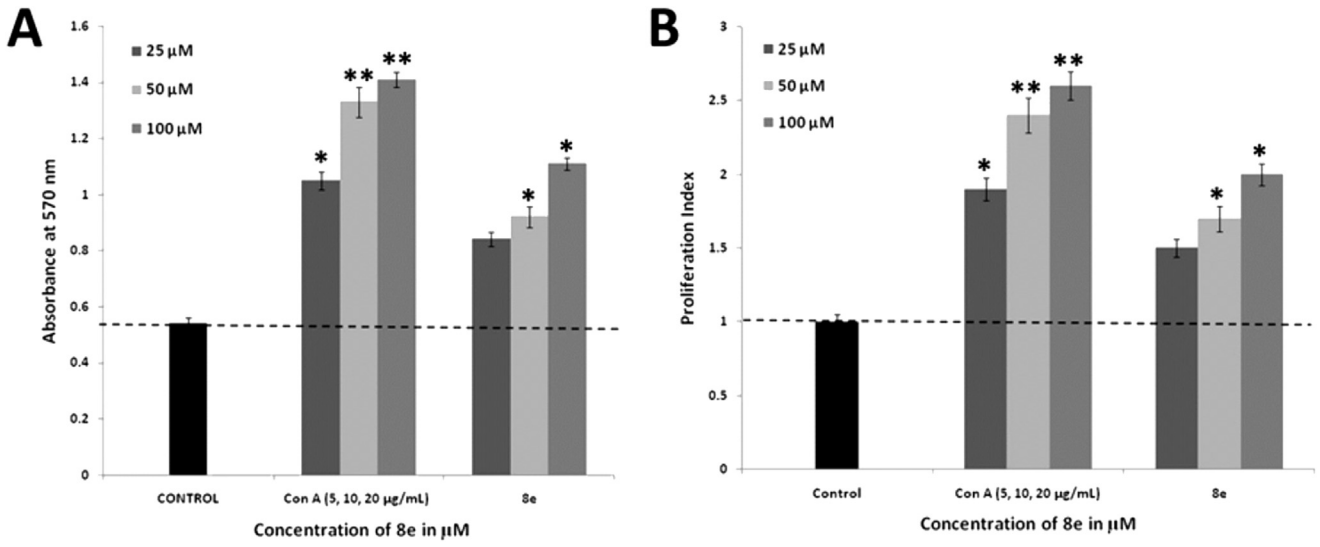


Fig. 1. Immunostimulatory activity of Compound **8e** against Human peripheral blood lymphocytes (PBLs) in-vitro. [**A**] Compound **8e** induces the proliferation of PBLs at 100 μM concentration, which is comparable to the reference mitogen Con A. [**B**] Proliferative Index of the Compound **8e** and Con A on human PBLs. Data represents results of three independent experiments. Values are mean ± SEM, n = 3, one way ANOVA followed by Tukey's multiple comparison test. Significant values are $^{*}p < 0.05$; $^{**}p < 0.01$.

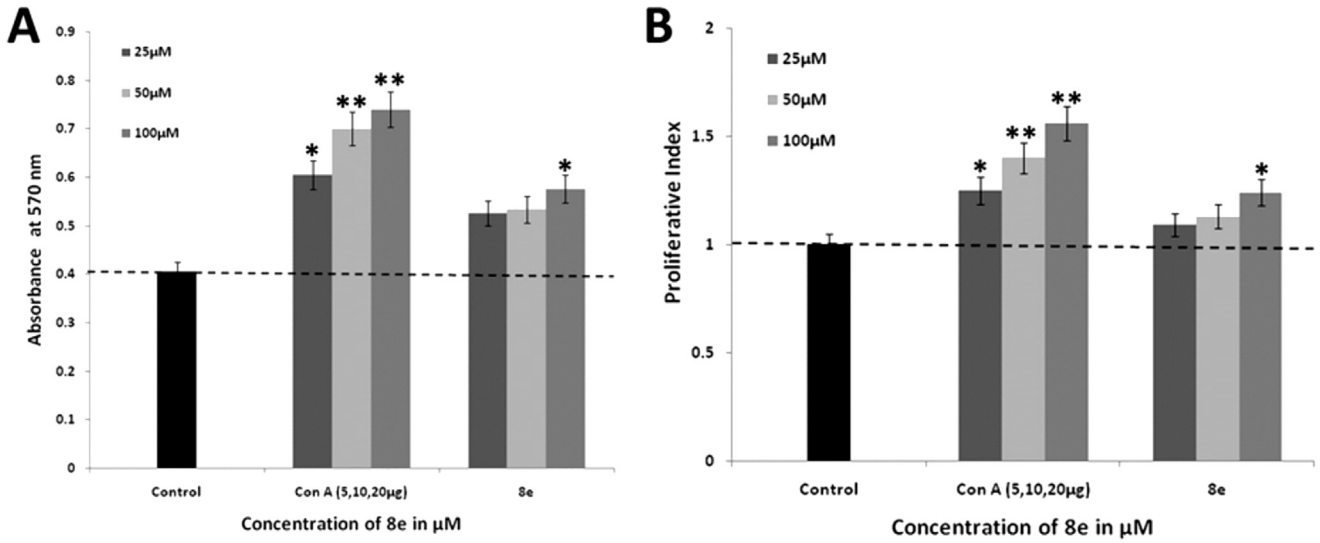


Fig. 2. Proliferative efficacy of Compound **8e** on murine splenocytes in-vitro. [**A**] Compound **8e** stimulates the proliferation of murine splenocytes at 100 μM concentration. Con A is used as positive control. [**B**] Proliferative Index of the Compound **8e** and Con A against murine splenocytes. Data represents results of three independent experiments. Values are mean ± SEM, n = 3, one way ANOVA followed by Tukey's multiple comparison test. Significant values are $^{*}p < 0.05$; $^{**}p < 0.01$.

*2.2.5. Pro-angiogenic activity of compound **8e***

Deregulated and deficient angiogenesis are implicated in various disorders, including retinopathies, impaired wound healing and immunity. As evidenced from the *in-vivo* Chick chorioallantoic membrane (CAM) and Rat corneal angiogenesis assay, compound **8e** was found to exhibit potent proangiogenic activity. Dense blood vessels were observed in the **8e** treated CAM at the concentration of 20 μM which was comparable with that of the VEGF induced angiogenesis. Similarly, thick belt of vascularisation was also observed in the rat cornea treated with compound **8e**. Key events involved in angiogenesis are endothelial cell activation, migration, proliferation, and tubule formation. Hence the observation of improved corneal blood vessels in **8e** treated group indicates that the compound could have induced the proliferation of endothelial cells, the key events involved in it, thereby accelerating the angiogenesis. The compounds immunostimulatory activity could also be attributed to its proangiogenic efficacy in *in-vivo* rat corneal assay, as lymphocytes and IL-2 and IL-23 are also known to induce the angiogenic process [25,30](Fig. 5).

3. Conclusion

The present study elucidated the synthesis of series of salicylic acid conjugated isoxazoline moiety **(8a-j),** which were then evaluated for their immunopharmacological activity targeting immune cell proliferation and angiogenesis. Considering all the above observations, it is very obvious that compound **8e** shows immunostimulatory activity towards human and murine splenocytes by triggering IL-2. The compound **8e** also shows pro-angiogenic activity as observed by *in-vivo* CAM and rat corneal angiogenesis

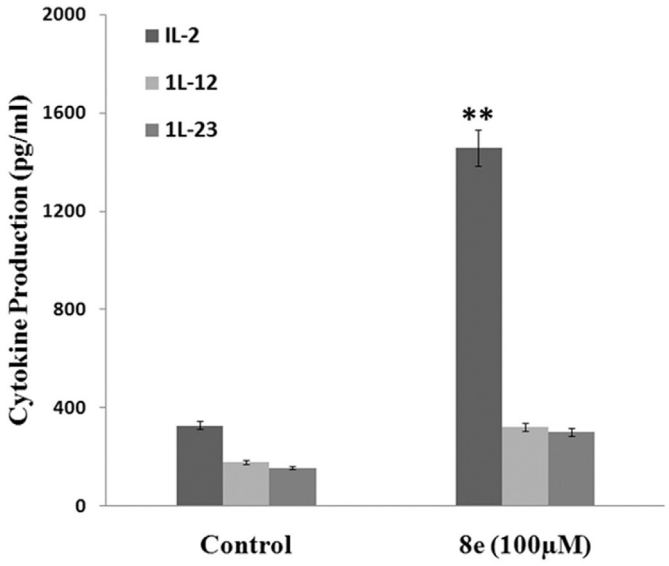


Fig. 3. Effect of compound **8e** on ex-vivointerleulin production: Human peripheral blood lymphocytes were treated with or without compound **8e** (100 μM). The supernatant media was assayed for IL-2/IL-12/IL-23 secretion by ELISA. The results were means of three determinations each conducted in triplicates.

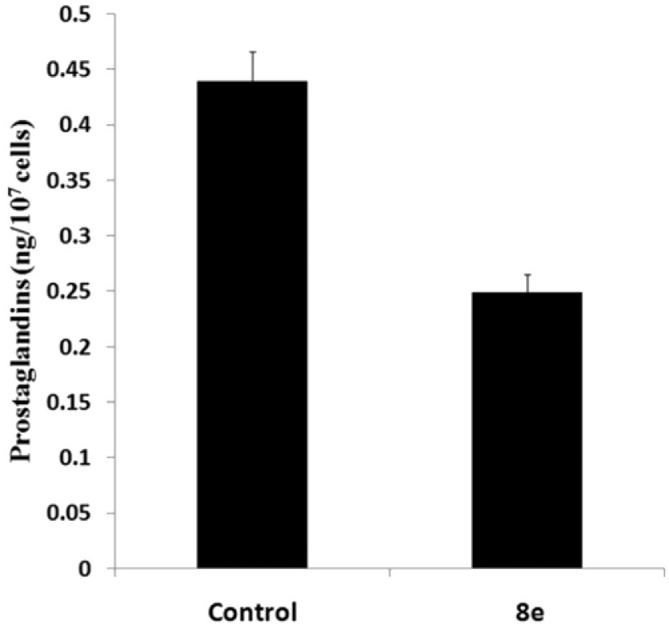


Fig. 4. Effect of compound **8e** on COX expression: Human peripheral blood lymphocytes were treated with or without compound **8e** for 72 h and cell lysate were assessed for COX-1 measurement. The results were means of three determinations each conducted in triplicates.

assay. But, a further study is required to understand its mechanism of action and the molecular signaling involved in the lymphocyte proliferation and angiogenesis induced by compound **8e**.

4. Materials and methods

4.1. Chemistry

Chemicals were purchased from Aldrich Chemical Co. TLC was performed on aluminum-backed silica plated with visualization by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. The IR spectra was recorded by the KBr pallet method on FT-IR Shimadzu 8300 spectrophotometer, NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer in DMSO and chemical shifts were recorded in parts per million down field from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrophotometer and important fragments are given with the relative intensities in the brackets. Elemental analysis results are within 0.4% of the calculated value.

4.1.1. Synthesis of 2-acetoxybenzoic acid **(2)**

To the stirring solution of salicylic acid **1** (28 mmol) in dry dichloromethane (25 mL), triethylamine (42 mmol) was added at 0 °C and stirred for 30 min. Acetyl chloride (28 mmol) was added drop wise and the resultant mixture was stirred at room temperature. After completion of the reaction which was monitored by TLC, the organic layer was washed with 5% hydrochloric acid (3 × 15 mL), followed by distilled water (15 mL) and brine solution (10 mL). The solvent was dried over anhydrous sodium sulfate and was evaporated under reduced pressure to obtain compound **2**. The crude compound was further purified by recrystallization with ethanol [31].

Yield 86%, m.p 138–140 °C; IR (KBr, γ/cm^{-1}): 3409–3490 (COOH), 3050 (CH),1730 (CO of COOH), 1675 (CO of $OCOCH_3$): ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.8 (s, 3H, CH_3), 7.1–8.1 (m, 4H, ArH), 11.5 (s, 1H, COOH). ^{13}C NMR: 25, 124, 125, 129, 135, 138, 150, 168, 169. LCMS (M^+): (180) Anal. Calcd. for: $C_9H_8O_4$: C, 60.00; H, 4.48; Found: C, 60.01; H, 4.56%.

4.1.2. Synthesis of 5-acetyl-2-hydroxybenzoic acid **(3)**

2-Acetoxybenzoic acid (**2,** 16 mmol) and anhydrous aluminum chloride (32 mmol) were heated to 120 °C and this temperature was maintained for 20 min. The reaction mixture was cooled down to room temperature and the complex was cleaved using 10% hydrochloric acid and the product was filtered, washed thoroughly with water (15 mL × 3) and recrystallized with ethanol to afford compound **3** as a pale yellow solid [32]. Yield 78%, m.p 142–144 °C; IR (KBr, γ/cm^{-1}): 3510–3610 (OH), 3414–3500 (OH of COOH), 1708–1716 (CO of COOH), 1732–1738 (CO of COCH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.3 (s, 3H, CH_3), 7.1–8.1 (m, 3H, ArH), 10.6 (s, 1H, OH), 11.5 (s, 1H, COOH). ^{13}C NMR: δ 25.34, 124.26, 125.42, 129.21, 135.45, 138.24, 150.32, 168.26. LCMS (M^+): (180) Anal. Calcd. for: $C_9H_8O_4$. C, 60.00; H, 4.48; Found: C, 60.08; H, 4.50%.

4.1.3. General procedure for synthesis of 5-(3-phenylacryloyl) 2-hydroxybenzoic acid**(5a-f)**

To a stirring solution of 2-acetoxybenzoic acid **(3,** 13 mmol) in ethanol, benzaldehyde (**4a,** 13 mmol) was added, followed by the addition of 10 mL of 30% sodium hydroxide drop wise. After complete addition, the reaction mixture was kept in the freezer overnight, the reaction mixture was diluted with 25 mL of water and neutralized with 10% hydrochloric acid to give yellow solid which was further purified by recrystallization with ethanol to get **5a** in 86% yield. The compounds **5b-f** were synthesised in the similar manner [33].

*4.1.3.1. 5-(3-Phenylacryloyl) 2-hydroxybenzoic acid **(5a)**.* Yield 86%, m.p. 159-161 °C; IR (KBr, γ/cm^{-1}): 3520–3615 (OH), 3408–3515 (OH of COOH), 3060 (CH), 1635 (C=C), 1718 (C=O of COOH),1685 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.1–8.1 (m, 8H, ArH), 7.3 (d, 1H, J = 8.3 Hz, H–C–C=O), 7.7 (d, 1H, J = 8.3 Hz, C=CH–Ar), 10.2 (s, 1H, OH), 11.6 (s, 1H, COOH). ^{13}C NMR: δ 114.22, 118.33, 122.13, 128.91, 129.35, 129.44, 129.37, 132.09, 135.33, 136.31, 144.28, 164.34, 171.68, 187.04. LCMS (M^+): (299), (121), (120). Anal. Calcd. for:

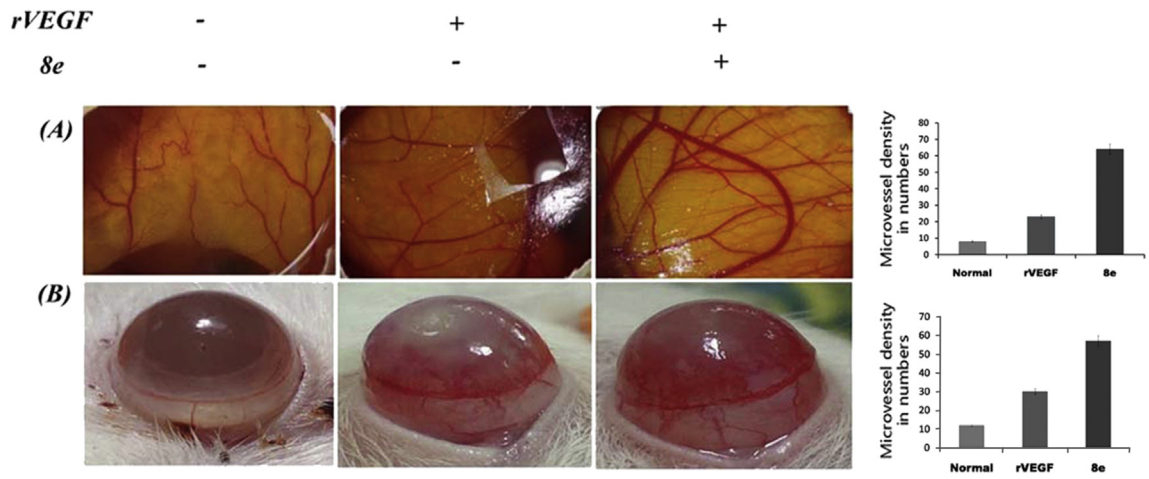


Fig. 5. Pro-angiogenic activity of Compound **8e** in in-vivo angiogenesis model systems [**A**] The images of in-vivo Chorioallontoic membrane (CAM) assay reveal that compound **8e** has significantly increased the vasculature when compared with the control. [**B**] Rat Corneal micro pocket assay implicated that compound **8e** showed an enormous increase in angiogenesis. The normal cornea shows less number of blood vessels, whereas the rVEGF and **8e** treated group shows a marked increase in neovasculature. The graphs depict increased neovascularization as quantified by Microvascular Density (MVD) count.

$C_{16}H_{12}O_4$: C, 71.64; H, 4.51; Found: 71.63; H, 4.48%.

*4.1.3.2. 5-(3-(4-Bromophenyl)acryloyl)2-hydroxybenzoic acid (**5b**).* Yield 74%, m.p. 153–156 °C; IR (KBr, γ/cm^{-1}): 3410–3510 (OH of COOH), 3510–3610 (OH), 3050 (CH), 1655 (C═C), 1705 (C═O of COOH), 1680 (C═O). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.1–8.1 (m, 7H, ArH), 7.1 (d, 1H, *J* = 8.4 Hz, H–C–C═O), 7.6 (d, 1H, *J* = 8.4 Hz, C═CH–Ar), 10.2 (s, 1H, OH), 11.7 (s, 1H, COOH). ^{13}C NMR: 114.82, 117.23, 123.22, 128.41, 129.46, 129.87, 130.95, 131.98, 134.83, 137.01, 144.65, 165.74, 171.58, 186.94. LCMS (M$^+$) (348), (346), (120). Anal.Calcd. for: $C_{16}H_{11}BrO_4$: C, 55.36; H, 3.19; found C, 55.38; H, 3.21%.

*4.1.3.3. 5-(3-(4-Hydroxyphenyl)acryloyl)2-hydroxybenzoic acid (**5c**).* Yield 60%, m.p.162–165 °C; IR (KBr, γ/cm^{-1}): 3405–3490 (OH of COOH), 3512–3600 (OH), 3050 (CH), 1659 (C═C), 1720 (C═O of COOH), 1670 (C═O). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.1–8.1 (m, 7H, ArH), 7.2 (d, 1H, *J* = 8.2 Hz, H–C–C═O), 7.7 (d, 1H, *J* = 8.2 Hz, C═CH–Ar), 10.8 (s, 1H, OH), 10.6 (s, 1H, OH), 11.7 (s, 1H, COOH).^{13}C NMR: 114.62, 118.03, 123.32, 128.01, 129.93, 129.67, 130.56, 131.89, 135.23, 136.51, 144.35, 165.34, 171.68, 187.44. LCMS (M+) (285), (121), (120). Anal.Calcd. for: $C_{16}H_{12}O_5$: C, 67.60; H, 4.25; found C, 67.30; H, 4.29%.

*4.1.3.4. 5-(3-(3-Hydroxyphenyl)acryloyl)2-hydroxybenzoic acid (**5d**).* Yield 92%, m.p. 172–175 °C; IR (KBr, γ/cm^{-1}): 3510–3610 (OH), 3411–3500 (OH of COOH), 3050 (CH), 1645 (C═C), 1710 (C═O of COOH), 1660 (C═O). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.1–8.1 (m, 7H, ArH), 7.0 (d, 1H, *J* = 8.4 Hz, H–C–C═O), 7.8 (d, 1H, *J* = 8.4 Hz, C═CH–Ar) 10.4 (s, 1H, OH), 11.5 (s, 1H, COOH). ^{13}C NMR: 114.08, 117.93, 122.12, 128.91, 129.25, 129.46, 129.87, 131.09, 135.13, 136.31, 144.35, 165.44, 171.78, 187.44. LCMS (M$^+$): (302), (304), (121), (120). Anal.Calcd. for: $C_{16}H_{11}ClO_4$: C, 63.48; H, 3.66; Found:C, 63.53; H, 3.62%.

*4.1.3.5. 5-(3-(4-Methoxyphenyl)acryloyl)2-hydroxybenzoic acid **(5e)**.* Yield 73%, m.p. 163–166 °C; IR (KBr, γ/cm^{-1}): 3515–3600 (OH), 3418–3505 (OH of COOH), 3075 (CH), 1644 (C═C), 1712 (C═O of COOH), 1690 (C═O). ^{1}HNMR (400 MHz, DMSO-d$_6$): δ 3.1 (s, 3H, OCH_3) 7.1–8.1 (m, 7H, ArH), 7.3 (d, 1H, *J* = 8.3 Hz, H–C–C═O), 7.9 (d, 1H, *J* = 8.3 Hz, C═CH–Ar) 10.4 (s, 1H, OH), 11.5 (s, 1H, OH). ^{13}C NMR: 60.03, 114.28, 117.93, 122.23, 128.61, 129.05, 129.24, 129.57, 131.46, 135.33, 137.01, 144.28, 164.94, 171.68, 188.04. LCMS (M$^+$): (299). Anal. Calcd. for: $C_{17}H_{14}O_5$: C, 68.45; H, 4.73; Found: C, 68.48; H, 4.72%.

*4.1.3.6. 5-(3-(4-Chlorophenyl)acryloyl)2-hydroxybenzoic acid (**5f**).* Yield 86%, m.p.168–170 °C; IR (KBr, γ/cm^{-1}): 3510–3610 (OH), 3412–3500 (OH of COOH), 3050 (CH), 1650 (C═C), 1700 (C═O of COOH), 1680 (C═O) ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.1–8.1 (m, 7H, ArH), 7.2 (d, 1H, *J* = 8.1 Hz, H–C–C═O), 7.5 (d, 1H, *J* = 8.1 Hz, C═CH–Ar), 10.6 (s, 1H, OH), 11.5 (s, 1H, COOH). ^{13}C NMR: δ 114.06, 118.07, 122.12, 128.93, 129.36, 129.47, 131.05, 132.09, 135.13, 136.15, 144.25, 165.24, 171.78, 187.44. LCMS (M$^+$): (302) (304) (120). Anal. Calcd. for: $C_{16}H_{11}ClO_4$: C, 63.48; H, 3.66; Found:C, 63.82; H, 3.63%.

4.1.4. General procedure for the synthesis of substituted N-hydroxy-1-phenylmethanimine

Compound 4-chlorobenzaldehyde **(6a)** was dissolved in 10 mL of ethanol, an equimolar mixture of hydroxylamine hydrochloride and sodium acetate was added. The mixture was heated on the water bath for 30 min, cooled and poured into ice cold water, a white solid so formed is filtered, dried and purified by recrystallisazation by using ethanol to afford compound **7a** in 73% yield. The compounds **7b-d** were synthesized in the similar method [19].

*4.1.5. General procedure for the synthesis of 3(4-chlorophenyl)-4(3-hydroxy-4-carboxy benzoyl)-5(4-chlorophenyl)4,5-dihydro dihydroisoxazoline **(8a-j)***

To a mixture of compound **5a** (1.8 mmol), 5-(-3-phenylacryloyl) 2-hydroxybenzoic acid, **7a** (1.9 mmol) and N-hydroxy-1-phenylmethanimine in was dissolved in absolute alcohol and chloramine-T (2 mmol) was added and refluxed on the water both for 4 h. The reaction was monitored by TLC. After completion, of the reaction the solvent was evaporated under reduced pressure, the reaction mass was dissolved in dichloromethane and the product was extracted by 10% sodium bicarbonate. On neutralizing with 10% hydrochloric acid title compound **8a** was obtained as a white solid, which was further purified by column chromatography. The compounds **8b-j** were synthesized by the same procedure [19].

*4.1.5.1. 3(4-Chlorophenyl)-4(3-hydroxy-4-carboxy benzoyl)-5(4-chlorophenyl)4,5-dihydro dihydroisoxazoline (**8a**).* Yield 60%. m.p. 168–170 °C; IR (KBr, γ/cm^{-1}): 3690–3610 (OH), 3428–3530 (OH of COOH), 3080 (CH), 1704 (C═O of COOH), 1685 (C═O), 1248 (C–O),

1609 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.8 (d, J = 8.3 Hz, 1H, C–CH–Ar), 6.6 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.0–7.8 (m, 11H, ArH) 10.6 (s, 1H, OH), 11.7 (s, 1H, COOH). ^{13}C NMR: 188.41, 169.21, 163.20, 144.24, 136.38, 135.12, 134.17, 133.21, 131.34, 129.38, 129.21, 128.32, 122.23, 119.32 118.02, 114.31, 113.46, 80.44, 60.61. LCMS (M^+): (456), (458), (460) Anal. Calcd. for: $C_{23}H_{15}Cl_2NO_5$: C, 60.54; H, 3.31; N, 3.07. Found: C, 60.52; H, 3.32; N, 3.07%.

*4.1.5.2. 3(4-Chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-bromophenyl)4,5-dihydrodihydroisoxazoline (**8b**).* Yield 72%, m.p. 172–175 °C: IR (KBr, γ/cm^{-1}): 3710–3630 (OH), 3425–3534 (OH of COOH), 1710 (C=O of COOH), 1670 (C=O), 1238 (C–O) 1612 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.6 (d, J = 8.2 Hz, 1H, C–CH–Ar), 6.8 (d, J = 8.2 Hz, 1H, H–C–C=O), 7.0–7.8 (m, 11H, ArH) 10.7 (s, 1H, OH), 12.3 (s, 1H, COOH). ^{13}C NMR 186.91, 177.02, 165.10, 143.04, 137.08, 136.34, 132.79, 131.94, 129.34, 128.83, 126.21, 122.34, 120.23, 118.34, 117.32, 116.02, 113.04, 80.53, 59.62. LCMS (M^+): (500), (502), (504) Anal. Calcd. for: $C_{23}H_{15}BrClNO_5$: C, 55.17; H, 3.02; N, 2.80. Found:C, 55.11; H, 3.03; N, 2.82%.

*4.1.5.3. 3(3-Chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-bromophenyl)4,5-dihydrodihydroisoxazoline (**8c**).* Yield 82%, m.p. 158–171 °C; IR (KBr, γ/cm^{-1}): 3700–3640 (OH), 3412–3528 (OH of COOH), 1708 (C=O of COOH), 1686 (C=O), 1236 (C–O) 1614 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.5 (d, J = 8.3 Hz, 1H, C–CH–Ar), 6.1 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.0–8.0 (m, 11H, ArH) 10.9 (s, 1H, OH), 11.7 (s, 1H, COOH). ^{13}C NMR 187.14, 174.52, 162.30, 145.42, 137.83, 136.12, 134.32, 133.09, 132.34, 131.35, 126.38, 125.21, 124.23, 123.13, 122.61, 122.10, 120.13, 119.12, 115.34, 81.54, 61.5. LCMS: (M^+) (500), (502), (504) Anal. Calcd. for:$C_{23}H_{15}BrClNO_5$: C, 55.17; H, 3.02; N, 2.80. Found: C, 55.13; H, 3.02; N, 2.88%.

*4.1.5.4. 3(3-Methoxyphenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-hydroxyphenyl)4,5-dihydrodihydroisoxazoline (**8d**).* Yield 82%, m.p. 158–171 °C; IR (KBr, γ/cm^{-1}): 3690–3640 (OH), 3422–3530 (OH of COOH), 1706 (C=O of COOH), 1680 (C=O), 1249 (C–O) 1616 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.9 (s, 3H), 5.3 (d, J = 8.3 Hz, 1H, C–CH–Ar), 6.9 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.0–8.0 (m, 11H, ArH), 10.4 (s, 1H, OH), 10.8 (s, 1H, OH), 11.7 (s, 1H, COOH). ^{13}C NMR: 186.24, 168.52, 162.30, 146.22, 138.38, 137.12, 136.12, 134.29, 132.32, 131.94, 127.38, 126.26, 125.23, 124.53, 121.36, 120.42, 116.42, 82.54, 63.56, 62.23. LCMS: (M^+) (433) Anal. Calcd. for: $C_{24}H_{19}NO_7$: C, 66.51; H, 4.42; N, 3.23. Found: C, 66.51; H, 4.42; N, 3.32%.

*4.1.5.5. 3(4-Methoxyphenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(3-chlorophenyl)4,5-dihydrodihydroisoxazoline (**8e**).* Yield 73%, m.p. 163–166 °C; IR (KBr, γ/cm^{-1}): 3710–3640 (OH), 3421–3526 (OH of COOH), 1706 (C=O of COOH), 1683 (C=O), 1240 (C–O) 1624 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.9 (s, 3H, OCH_3), 5.4 (d, J = 8.2 Hz, 1H, C–CH–Ar), 6.2 (d, J = 8.2 Hz, 1H, H–C–C=O), 7.0–8.0 (m, 11H, ArH), 10.6 (s, 1H, OH), 12.1 (s, 1H, COOH). ^{13}C NMR, 185.26, 167.53, 163.31, 145.22, 138.21, 137.11, 136.42, 135.39, 134.12, 132.94, 128.38, 127.23, 125.26, 123.32, 122.12 121.23, 120.42, 117.52, 116.31, 83.53, 65.46, 63.23. LCMS: (M^+) (452), (454) Anal. Calcd. for: $C_{24}H_{18}ClNO_6$ C, 63.79; H, 4.02; N, 3.10. Found: C, 63.73; H, 4.01; N, 3.10%.

*4.1.5.6. 3(4-Hydroxyphenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(3-chlorophenyl)4,5-dihydrodihydroisoxazoline (**8f**).* Yield 77%, m.p. 184–186 °C: IR (KBr, γ/cm^{-1}), 3710–3640 (OH): 3465–3525 (OH of COOH), 1702 (C=O of COOH), 1684 (C=O), 1241 (C–O), 1615(C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.3 (d, J = 8.3 Hz, 1H, C–CH–Ar), 6.4 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.0–8.0 (m, 11H, ArH) 10.3 (s, 1H, OH), 10.6 (s, 1H, OH), 12.1 (s, 1H, COOH). ^{13}CNMR, 186.26, 166.53, 163.12, 162.41, 144.32, 137.24, 139.12, 137.12, 135.18, 134.93, 133.04, 129.62, 128.24, 127.38, 124.52, 123.43, 121.02, 120.54, 116.25, 82.03, 66.46. LCMS: (M^+) (438), (440). Anal. Calcd. for: $C_{23}H_{16}ClNO_6$: C, 63.09; H, 3.68; N, 3.20; Found: C, 63.19; H, 3.64; N, 3.23%.

*4.1.5.7. 3(3-Chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(3-chlorophenyl)4,5-dihydrodihydroisoxazoline (**8g**).* Yield 82%. m.p. 185–188 °C; IR (KBr, γ/cm^{-1}): 3690–3620 (OH), 3430–3522 (OH of COOH), 1700 (C=O of COOH), 1687 (C=O), 1239(C–O) 1616(C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.2 (d, J = 8.3 Hz, 1H C–CH-Ar), 6.5 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.0–8.0 (m, 11H, ArH), 10.6 (s, 1H, OH), 11.1 (s, 1H, COOH). ^{13}C NMR: 187.36, 167.43, 163.41, 145.23, 139.12, 138.33, 136.82, 135.39, 134.04, 132.23, 130.22, 128.48, 126.32, 124.21, 124.52, 123.22, 122.02, 121.03, 118.32, 115.25, 83.03, 67.46. LCMS: (M^+) (455), (457), (459). Anal. Calcd. for: $C_{23}H_{15}Cl_2NO_5$: C, 60.54; H, 3.31; N, 3.07; Found: C, 60.52; H, 3.33; N, 3.04%.

*4.1.5.8. 3(4-Chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-methoxyphenyl)4,5-dihydrodihydroisoxazoline (**8h**).* Yield 73%. m.p. 154–157 °C; IR (KBr, γ/cm^{-1}): 3720–3620 (OH), 3425–3520 (OH of COOH), 1707 (C=O of COOH), 1682 (C=O), 1245 (C–O) 1626 (C=N). ^{1}H NMR (400 MHz, DMSO-d^6): δ 3.1 (s, 3H, OCH_3), 5.2 (d, J = 8.3 Hz, 1H, C–CH–Ar), 6.0 (d, J = 8.3 Hz, 1H, H–C–C=O), 7.1–7.9 (m, 11H, ArH), 10.7 (s, 1H, OH), 12.2 (s, 1H, COOH). ^{13}C NMR: 184.36, 168.53, 164.13, 146.21, 139.61, 137.32, 138.21, 134.49, 133.54, 132.43, 126.31, 127.83, 124.36, 123.42, 122.34, 121.22, 118.52, 84.53, 62.43, 65.23. LCMS: (M^+) (452), (454). Anal. Calcd. for: $C_{24}H_{18}ClNO_6$: C, 63.79; H, 4.02; N, 3.10. Found: C, 63.63; H, 4.10; N, 3.12%.

*4.1.5.9. 3(3-Chlorophenyl)-4(3-hydroxy-4-carboxybenzoyl)-5(4-methoxyphenyl)4,5-dihydrodihydroisoxazoline (**8i**).* Yield 73%, m.p. 154–157 °C; IR (KBr, γ/cm^{-1}): 3700–3620 (OH), 3400–3585 (OH of COOH), 1703 (C=O of COOH), 1687 (C=O), 1239 (C–O) 1620 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.0 (s, 3H, OCH_3), 4.9 (d, J = 8.2 Hz, 1H, C–CH–Ar), 6.3 (d, J = 8.2 Hz, 1H, H–C–C=O), 7.1–8.0 (m, 11H, ArH), 10.5 (s, 1H, OH), 11.9 (s, 1H, COOH). ^{13}C NMR, 185.346, 165.33, 165.23, 147.31, 140.61, 137.21, 133.29, 132.54, 128.83, 128.10, 127.34, 126.41, 125.26, 124.32, 123.48, 123.05, 122.32, 120.23, 119.42, 85.43, 67.27, 63.53. LCMS: (M^+) (452), (454). Anal. Calcd. for: $C_{24}H_{18}ClNO_6$: C, 63.79; H, 4.02; N, 3.10. Found: C, 63.76; H, 4.08; N, 3.19%.

*4.1.5.10. 3-Phenyl-4(3-hydroxy-4-carboxybenzoyl)-5(2,4-dimethoxyphenyl)4,5-dihydrodihydroisoxazoline (**8j**).* Yield 73%, m.p. 154–157 °C; IR (KBr, γ/cm^{-1}): 3700–3620 (OH), 3410–3588 (OH of COOH), 1704 (C=O of COOH), 1675 (C=O), 1239 (C–O) 1620 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.8 (s, 6H, OCH_3), 5.8 (d, J = 8.2 Hz, 1H, C–CH–Ar), 6.7 (d, J = 8.2 Hz, 1H, H–C–C=O), 7.1–8.0 (m, 11H, ArH), 10.5 (s, 1H, OH), 11.9 (s, 1H, COOH). ^{13}CNMR, 185.34, 165.33, 165.23, 147.31, 140.61, 137.21, 135.62, 134.43, 133.29, 133.09, 132.54, 128.83, 127.45, 125.26, 123.48, 122.32, 120.45, 118.42, 117.32, 85.43, 67.27, 63.53. LCMS: (M^+) (448), Anal. Calcd. for: $C_{25}H_{21}NO_7$: C, 67.11; H, 4.73; N, 3.13. Found: C, 67.21; H, 4.70; N, 3.11%.

4.2. Biology

4.2.1. Chemicals and reagents

[Concanavalin A (Con A)] Ficoll-hypaque, tissue culture grade sodium bicarbonate and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide A.R.] were procured from Sigma–Aldrich Co., St. Louis, MO, USA. RPMI-1640 medium, Penstrip and Fetal bovine serum (FBS) were purchased from Gibco-Invitrogen, USA. Cell culture plates (96 well) were products of eppendorf, Germany. Anti-Interleukin antibodies for IL-2, IL-12 and IL-23 were procured from Merck Millipore. The COX assay kit was from Cayman chemicals. All other chemicals and reagents used in the experiments were of analytical grade.

4.2.2. Animal and ethics

Healthy Swiss Albino male mice weighing 25 ± 2.0 g are housed in polyacrylic cages and maintained under standard conditions (25 ± 2 °C) with 12 ± 1 h dark/light cycle. All procedures for animal experimentation were approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, Karnataka, India (NCP/IAEC/CL/101/05/2012-13); informed consent was obtained from all human volunteers for obtaining peripheral venous blood in the age range of 18–60 years.

4.2.3. Cell culture and maintenance

HEK293 cells were cultured at 37 °C in incubator supplemented with 5% CO_2. The cells were treated using increasing concentrations of compounds **8a-j** (0, 10, 20, 50, 100 μM in DMSO) at various time intervals (0–72 h) and further used for experiments. Appropriate vehicle control and ConA as positive control were used and each experiment was repeated a minimum of 3 independent times.

4.2.4. Isolation of human peripheral blood lymphocytes (PBLs)

Ten milliliters of heparinized venous blood was layered carefully on Ficoll-hypaque (density = 1.077 g/mL) contained in a polystyrene tube. The tubes were centrifuged at 250 × g at 25 °C for 20 min. After centrifugation, below the plasma layer, a circle of white translucent coat containing lymphocytes was aspirated carefully using a Pasteur pipette. The cells were then resuspended in isotonic phosphate-buffered saline (PBS) and mixed by gentle aspiration. The buffy coat containing lymphocytes was washed 4–5 times using PBS at 4 °C, and finally were placed in complete RPMI-1640 medium [SNP].

4.2.5. Isolation of murine splenocytes

Murine splenocytes were isolated in accordance with the earlier described methods with slight modifications [34]. Briefly, spleen was collected under aseptic conditions from normal Swiss albino mice (23–25 g, 12-weeks-old) after sacrificing, placed in isotonic phosphate buffered saline. These tissues were separately minced and passed through a fine steel mesh to obtain a homogenous cell suspension. The cells were pelleted to remove the tissue debris. After centrifugation (380 × g at 4 °C for 10 min), the pelleted cells were washed three times with PBS (400 × g at 4 °C for 10 min). The pellet obtained after washing with PBS was resuspended in a modified ammonium chloride buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.4 containing 10 mM sodium edetate) and incubated at 4 °C for 5 min to remove the erythrocytes [34]. After the cells were centrifuged at 400 × g at 4 °C for 20 min, the pellet was gently resuspended in HBSS and washed three times in the same buffer, and finally resuspended in RPMI-1640 medium.

4.2.6. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] based on the method described previously with slight modifications [34]. Briefly, the human peripheral blood lymphocytes (PBL's) and murine splenocytes were cultured separately in flat bottomed 96 wells microtiter plates (200 μL of 2.5 × 10^6 cells/mL), in a CO_2 incubator at 37 °C with 5% CO_2. After 3 h of incubation, compound **8e** in the increasing concentration range (0, 10, 20, 50, 100 μM in DMSO) was used to treat the cells. Similarly, the reference mitogen Con A was also treated in the concentration range (0, 1, 2, 5, 10, 20 μg/mL in 10 mM PBS) (Lymphocytes in the absence of compounds represent control, blank was carried out with complete medium only). After incubation for 72 h in the same conditions, 20 μL of 5 mg/mL MTT solution was added and incubated for further 4 h under the same conditions. After removing the plates, the samples were aspirated to an eppendorf tube and centrifuged at 750 × g at 4 °C for 15 min. Supernatant was removed and the blue formazan crystals were resolubilized in 200 μL in DMSO. After dissolving the crystals, 100 μL of each sample was taken in microtiter plates. Plates were read in a microtitreplate reader at 570 nm.

4.2.7. Interleukin measurement assay

Human peripheral blood lymphocytes (5 × 10^5 cells/well on 24-well plates) were cultured in RPMI-1640 and stimulated with compound **8e** as described above. The plate was maintained at 37 °C in a humidified incubator with a 5% CO_2 air atmosphere for 72 h, followed by centrifugation for 10 min, at 240 × g and at room temperature. The cell supernatants were collected and assayed for IL-2/12/23 concentrations by using an enzyme-linked immunosorbent assay, according to the standard protocols using appropriate antibodies. The assays were conducted in triplicates.

4.2.8. Cyclooxygenase-1 (COX-1) activity assay

COX-1 activity was measured in human PBL's stimulated with compound **8e** in accordance with the previously described methods with slight modifications [29]. Briefly, after 24 h incubation post stimulation, cells were washed twice with icecold PBS and disrupted by sonication in icecold 100 mMTris-HCl, pH 7.8. COX enzymatic activity was determined with 100 mg of protein from cell sonicates in 0.2 mL volume of assay buffer (100 mM Tris-HCl (pH 7.8), 5 mM tryptophan, and 5 mM reduced glutathione). Samples were incubated at 37 °C for 15 min in the presence of an excess arachidonic acid (100 mM). The reaction was stopped by boiling, and samples were centrifuged at 10,000 × 3 *g* for 10 min. Concentrations of PGs were measured according to the manufacturer's instructions (Cayman Chemical).

4.2.9. Assay for angiogenesis

4.2.9.1. CAM assay. The *in-vivo* anti-angiogenic effect induced by $rVEGF_{165}$ was analyzed following treatment with compound **8e** (8 μM) in 12 days, fertilized egg CAM as described earlier [35] and changes in the MVD was analyzed and photographed using Sony steady shot DSC-W610 camera.

4.2.9.2. Rat corneal assay. The effect of compound **8e** in neovessel formation is assessed by rat corneal micro pocket assay as described previously [36,37]. Briefly, hydron polymer (poly-hydroxyethyl-methacrylate (poly HEMA), Sigma Aldrich, USA) was dissolved in ethanol to a final concentration of 12%. All aliquots of this mixture was then pipetted on to the teflon sheet, Saline (group 1), 1 μg of $rVEGF_{165}$ (group 2), $rVEGF_{165}$ + Compound **8e** 20 μg (group 3) was added to each pellet and allowed to dry under a laminar flow hood at room temperature for 2 h. The pellets were incubated at 4 °C overnight. The corneal implantation of the pellet was done as described previously. The extent of vasculature and the number of blood vessels were quantified after the treatment.

4.3. Statistical analysis

Values were expressed as mean ± standard error (SEM). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student's t-test ($^*p < 0.05$) and ($^{**}p < 0.01$).

Conflict of interest statement

The authors hereby state that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.02.052.

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