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Research paper

Eugenol derived immunomodulatory molecules against visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a life threatening infectious disease caused by *Leishmania donovani*. It leads to the severe immune suppression in the host defense system. Higher cytotoxicity, rigorous side effects and lower therapeutic indexes (TI) of current antileishmanial drugs have created a necessity to develop new molecules with better antileishmanial activity and high TI value. In this study, we have synthesized 36 derivatives of eugenol and screened them for their activity against promastigote and amastigote forms of *L. donovani*. Among the synthesized derivatives, comp.**35** showed better antileishmanial activity against extra cellular promastigotes (IC_{50}- 20.13 ± 0.91 μM) and intracellular amastigotes (EC_{50}- 4.25 ± 0.26 μM). The TI value (82.24 ± 3.77) was found to improve by 10–13 fold compared to Amphotericin B and Miltefosine respectively. Treatment with comp.**35** (5 μg/ml) enhanced the nitric oxide (NO) generation, iNOS2 mRNA expression (~8 folds increase) and decreased the arginase-1 activity (~4 folds) in *L. donovani* infected peritoneal macrophages. Comp.**35** had also increased the IL-12 (~6 folds) and decreased the IL-10 (~3 folds) mRNA expression and release *in vitro*. Results of *in vivo* studies revealed that comp.**35** treatment at 25 mg/kg body weight efficiently cleared the hepatic and splenic parasite burden with enhanced Th1 response in *L. donovani* infected BALB/c mice. Hence, this study clearly represents comp.**35**, as an immunomodulatory molecule, can induce host protective immune response against visceral leishmaniasis through enhanced NO generation and Th1 response, which are essentials against this deadly disease.

1. Introduction

Visceral leishmaniasis (VL) is one of the most devastating infectious diseases caused by obligate intracellular parasites, *Leishmania donovani* with a worldwide incidence of 0.3 million new cases per year. It mainly affects the proper functioning of host immune system and increases the risk of concomitant infections [1]. The problems with contemporary medicines like Pentavalent antimonials, Amphotericin-B (AmpB), Pentamidine and Miltefosine are their low therapeutic index (TI), severe side effects, acute cytotoxicity, high cost and emergence of their resistance strains [2]. In the absence of effective vaccination against VL, we are compelled to rely only on the available drugs to cure this disease. Although new entities have been reported in the recent literatures which act upon the type IB DNA topoisomerase, and pteridine reductase 1 of *Leishmania* parasite [3–6]. Several other compounds are also reported which effectively arrest the parasite cell cycle and kill the parasite by concomitant nitrite generation in host cells [7,8].

There are two popular drug developmental approaches for VL treatment, (i) direct killing of the parasite and (ii) killing of the parasite by boosting the immune response of the host cell. It is already found that the second approach is very much effective compared to the first one in the sense of toxicity measure. For the clearance of the parasite inside the macrophage (host cell) specific cytokine responses need to be impaired like IL-10, TGF-β, and at the same time other cytokine response like IL-12, IFN-γ need to be enhanced. Similarly, *in vivo* model, leishmanial parasite enters into the host system and modulates the host immune system with

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strong Th2 cytokine response that causes severe immune suppression [9]. Therefore, it is quite challenging to develop such an immunomodulatory molecule for the treatment of VL.

In an attempt to develop a set of anti-visceral leishmanial immunomodulatory compounds inspired by natural products, we have selected eugenol as the parent scaffold. Eugenol, a phenolic phytochemical, is found as a major component of many essential oils extracted from various medicinal plants. It is known to have various medicinal properties, but the most intriguing one is its antileishmanial activity [10,11]. Eugenol rich essential oil from different plant sources has been documented to have high toxicity towards promastigotes and amastigotes of *L. amazonensis,* and *L. donovani.* [10,12] Simple chemical modifications of the eugenol scaffold by means of acetylation or benzoylation have also been found to improve the efficacy by 2 or 5 folds, respectively compared to eugenol (on *L. infantum* and *L. chagasi* promastigote) along with the reduction of their cytotoxicity level [13]. Hence the simple modification may further enhance the antileishmanial activity.

In this report, we have prepared several cyclic and acyclic derivatives of eugenol by means of suitable chemical transformation. After which, these derivatives were screened for their antileishmanial activity against promastigote and amastigote forms of *L. donovani*. The best comp.**35** was further studied to elucidate its immunomodulatory activity and mechanistic aspects of parasite killing in *L. donovani* infected peritoneal macrophages as *in vitro* model. Then the antileishmanial activity of comp.**35** in *L. donovani* infected BALB/c mice was studied by estimating the hepatic and splenic parasite burden and other immunomodulatory effector responses.

2. Results and discussion

2.1. Synthesis of eugenol derivative

In this study, we have synthesized three sets of eugenol derivatives using the phenolic OH of eugenol (**1**). For the first set of molecules (comp. **3** to **16**), we had connected different aliphatic chains with the parent molecule via an ether linkage. The ether linkage was prepared under Mitsunobu reaction condition for **3**, **4** and **7**. However, for the other molecules of this set we obtained a low yield, under the same reaction condition and it was because of the moisture present with the corresponding alcohols. Therefore, to overcome this problem and to reduce the reaction time, we prepared the remaining molecules by using alkyl/alkenyl halides in presence of NaH in quantitative yield (Scheme 1). Similarly, the second set of molecules (**17**–**35**) was prepared either by the reaction with anhydrides or the corresponding carboxylic acids. For the esterification two different coupling reagents DCC and EDCI.HCl were used. The selection of the coupling agent was made based on the yield and the ease of purification. For the aliphatic carboxylic acids EDCI.HCl gave us the best yield.

In an effort to introduce polar amino group we prepared third set of molecules where *N*-Boc-L-proline, *N*-Boc-isonipecotic acid and N_{α}-Boc-L-tryptophan were coupled with the eugenol moiety using DCC. However, under the usual condition at 5% TFA in DCM decomposition of the starting material was observed, thus, we used dry HCl gas in MeOH for the removal of Boc to yield **36**, **37** and **38** respectively (Scheme 1).

2.2. Structure activity relationship

The leishmanicidal activity of the total thirty six synthesized molecules were tested against both promastigote and amastigote form of *L. donovani*. The nitric oxide production which is primarily responsible for the killing of parasite within the host macrophage was determined for individual compounds and given in EC_{50} value. Finally the therapeutic index (TI) was calculated by the ratio of cytotoxicity with EC_{50}. Miltefosine and Amphotericin B were used as the reference drug candidate, whereas, eugenol (**1**) and methyleugenol (**2**) had been used as reference molecules.

2.2.1. Activity against promastigote

The activity (IC_{50}) of the first set of molecules (**3** to **16**) against promastigote form of *L. donovani* was found to be improved with the elongation of chain length compared to the reference molecules **1** (441.66 ± 13.28 μM) and **2** (475.45 ± 19.08 μM) (Table 1). The IC_{50} value for comp. **3** was obtained 237.58 ± 13.51, which was almost two folds better than the reference molecules. For the further elongation of the chain length upto C18 we observed a sharp decrease of IC_{50} values. However, a limiting condition was observed for comp.**5** (C2) and **8** (C5) to **16** (C18). The best activity was found for comp.**13** (49.92 ± 7.06 μM), with further elongation of the chain length the IC_{50} values were found to increase. This observation indicated that the C10 chain length was the most favoured selection. Similarly, for the second set of molecules (**17** to **35**), with aromatic substitution the IC_{50} values were found to be in the range of 82.38 ± 6.19 μM to 28.86 ± 1.14 μM and the best activity was observed for comp.**24** (28.86 ± 1.14 μM). The presence of three different electron withdrawing groups fluoro, chloro and nitro at different position of the aromatic ring did not showed appreciable effect on their antileishmanial activity. Nevertheless, increase of the spacer length (comp.**23** and **24**) showed a significant improvement in the activity against promastigotes. For the aliphatic substitution, the better activity was observed for comp.**32**, **33**, **34** and **35** with C9, C11, C15 and C17 chain length respectively (Table 2). Unlike comp.**16** the best activity was observed for **35** (20.13 ± 0.91 μM) with C17 chain length. The IC_{50} values for the third set of molecules were found in between 97.35 ± 3.90–52.97 ± 1.57 μM. Among the three others of this set comp.**38** showed the best activity (52.97 ± 1.57 μM) (Table 3).

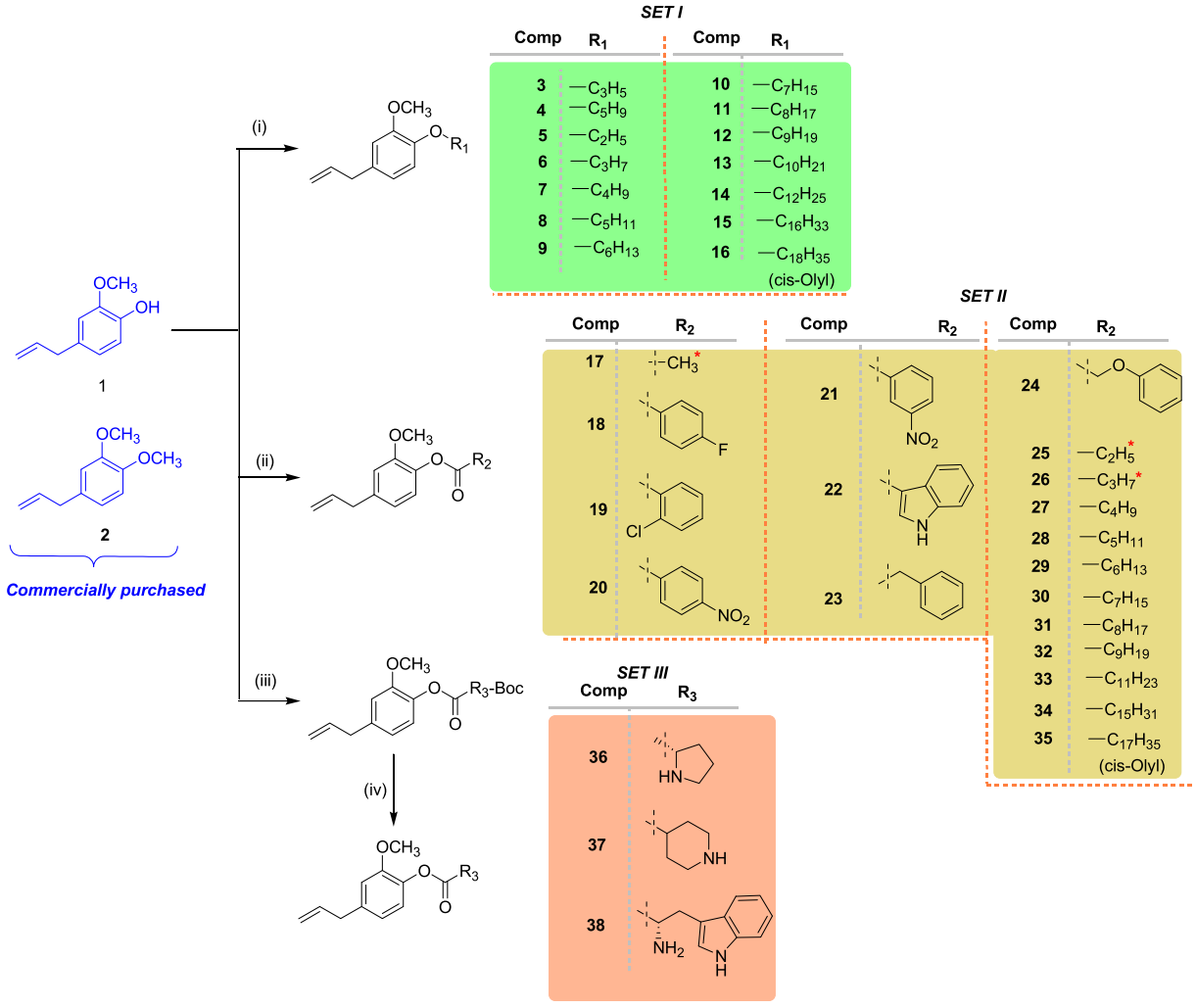
Our best molecule **35** showed two and twenty times higher IC_{50} value compared to the known available drugs Miltefosine and AmpB respectively. However, the TI value for **35** was three and two times better than the Miltefosine and AmpB respectively (Table 1). It is pertinent to note that for **35** the activity was compromised to a large extent with its toxicity.

1 = 50% Inhibitory concentration (IC_{50}); 2 = IC_{50} against RAW264.7 cells; 3 = Therapeutic index (TI); 4 = 50% Effective concentration (EC_{50}); 5 = EC_{50} against mouse peritoneal macrophages.

2.2.2. Activity against amastigote

The activity against amastigotes was determined to estimate their immunomodulatory activity. For the first set of molecules (**3** to **16**), EC_{50} values were found to be comparable with comp.**1** and **2**, except **9** to **15**. Unlike the IC_{50} values, EC_{50} values were found to decrease from comp.**8** with C5 chain length and the best activity was observed for **15** (17.06 ± 1.57 μM) with C16 chain length. The cytotoxicity for this set of compounds was found in the range of 393 to 90 μM, which is much lower than the known drugs Miltefosine and AmpB (Table 1). The calculated TI values for Miltefosine and AmpB were 6.34 ± 0.45 and 7.78 ± 0.59 respectively, whereas for **15** the TI value was found 14.47 ± 0.91.

For the second set of molecules with aromatic ring, the better activity (EC_{50}) was obtained for comp.**21** (26.24 ± 1.34 μM) and **22** (25.31 ± 1.07 μM) having the nitro group, although the nitric oxide generation was found to be comparable with **19** and **20**. Nevertheless, for comp.**23** and **24** the EC_{50} values were reduced to 9.28 ± 0.46 μM and 7.17 ± 0.50 μM respectively with the improved TI value (41.53 and 54.52 respectively). This enhanced activity



Scheme 1. Reagents and conditions: (i) R_1-OH, DIAD/PPh_3, THF, 7–8 h, rt, or alkyl/alkenyl halide, NaH, DMF, 3–4 h, rt; (ii) $(R_2CO)_2O^*$, Pyridine/$CHCl_3$, 6 h, rt, or R_2CO_2H, DCC/DMAP, DMF, 8–10 h, rt, or R_2CO_2H, EDCI.HCl/DMAP, DMF, 3 h, rt; (iii) N/Nα-Boc-R_3-CO_2H, DCC/DMAP, DMF, 6–8 h, rt and (iv) HCl gas (dry), MeOH, 3 h, rt.

Table 1
Antipromastigote and Antiamastigote activities against *L. donovani*, TI values and nitric oxide generation of eugenol and its ether derivatives (Compound 1 to 16 and Miltefosine &AmpB).

Comp.	Promastigote			Amastigote			NO generation (μM)
	[1]IC_{50} (μM)	[2]Cytotoxicity IC_{50} (μM)	[3]TI	[4]EC_{50} 20. (μM)	[5]Cytotoxicity EC_{50} (μM)	[3]TI	
1	441.66 ± 13.28	527.04 ± 14.62	1.19 ± 0.01	173.26 ± 7.37	475.76 ± 13.52	2.75 ± 0.07	7.45 ± 0.32
2	475.45 ± 19.08	517.42 ± 6.51	1.09 ± 0.05	203.28 ± 9.03	485.05 ± 18.46	2.39 ± 0.08	10.82 ± 0.78
3	237.58 ± 13.51	384.78 ± 16.74	1.64 ± 0.15	120.28 ± 5.34	353.36 ± 15.76	2.95 ± 0.12	13.86 ± 0.68
4	242.08 ± 5.47	358.51 ± 8.65	1.48 ± 0.07	157.20 ± 7.36	371.94 ± 4.69	2.38 ± 0.08	8.37 ± 0.85
5	71.67 ± 5.83	101.79 ± 5.05	1.43 ± 0.08	162.13 ± 12.69	90.50 ± 9.31	0.55 ± 0.05	10.43 ± 1.03
6	133.07 ± 9.02	110.04 ± 6.98	0.83 ± 0.066	155.71 ± 9.26	110.72 ± 6.40	0.71 ± 0.03	10.64 ± 0.59
7	308.93 ± 20.65	328.67 ± 4.04	1.07 ± 0.05	147.15 ± 4.90	345.06 ± 10.71	2.35 ± 0.07	14.23 ± 0.92
8	90.81 ± 5.55	178.37 ± 7.04	1.96 ± 0.04	83.38 ± 6.14	163.87 ± 9.17	1.97 ± 0.04	12.95 ± 1.56
9	80.28 ± 6.28	291.63 ± 23.31	3.63 ± 0.11	36.40 ± 4.15	272.26 ± 18.16	7.60 ± 0.41	20.81 ± 1.70
10	61.32 ± 8.65	197.79 ± 20.06	3.28 ± 0.14	41.08 ± 5.37	197.15 ± 20.66	4.88 ± 0.34	22.43 ± 1.82
11	62.30 ± 6.98	357.14 ± 22.14	5.85 ± 0.39	48.69 ± 6.26	392.66 ± 30.86	8.30 ± 0.81	26.01 ± 2.21
12	81.94 ± 8.81	332.07 ± 23.69	4.27 ± 0.73	51.78 ± 6.13	326.29 ± 15.18	6.47 ± 0.48	25.19 ± 1.01
13	49.92 ± 7.06	411.73 ± 22.24	8.61 ± 0.95	28.97 ± 3.48	272.37 ± 23.15	9.67 ± 0.91	26.17 ± 1.45
14	52.51 ± 4.87	299.34 ± 16.00	5.76 ± 0.23	25.98 ± 2.74	292.43 ± 23.19	11.41 ± 0.74	28.12 ± 1.55
15	58.05 ± 1.62	291.69 ± 7.90	5.02 ± 0.02	17.06 ± 1.57	250.93 ± 6.51	14.47 ± 0.91	29.1 ± 1.26
16	60.24 ± 3.16	423.80 ± 30.89	7.04 ± 0.42	25.51 ± 2.94	393.66 ± 23.00	15.94 ± 1.79	28.94 ± 1.95
Milte	10.38 ± 0.32	44.78 ± 0.98	4.3 ± 0.21	6.40 ± 0.42	40.66 ± 2.18	6.34 ± 0.45	26.01 ± 2.85
AmpB	1.36 ± 0.16	10.02 ± 0.45	7.4 ± 0.58	1.18 ± 0.13	9.18 ± 0.85	7.78 ± 0.59	n.d.

1 = 50% Inhibitory concentration (IC_{50}); 2 = IC_{50} against RAW264.7 cells; 3 = Therapeutic index (TI); 4 = 50% Effective concentration (EC_{50}); 5 = EC_{50} against mouse peritoneal macrophages.

Table 2
Antipromastigote and Antiamastigote activities against *L. donovani*, TI values and nitric oxide generation of ester derivatives of eugenol (compound 17 to 35).

Comp.	Promastigote			Amastigote			NO generation (μM)
	[1]IC_{50} (μM)	[2]Cytotoxicity IC_{50} (μM)	[3]TI	[4]EC_{50} (μM)	[5]Cytotoxicity EC_{50} (μM)	[3]TI	
17	165.92 ± 10.57	356.09 ± 20.32	2.15 ± 0.06	99.11 ± 5.38	352.21 ± 6.64	3.59 ± 0.21	12.44 ± 1.84
18	61.26 ± 1.71	363.85 ± 17.32	5.93 ± 0.12	78.41 ± 9.78	400.00 ± 5.13	5.24 ± 0.45	14.56 ± 1.01
19	64.48 ± 2.97	251.10 ± 4.36	3.91 ± 0.13	57.94 ± 3.14	275.01 ± 4.26	4.79 ± 0.29	14.58 ± 1.14
20	82.38 ± 6.19	260.57 ± 7.63	3.18 ± 0.15	26.68 ± 1.69	244.26 ± 8.75	9.21±0.69	27.28 ± 2.69
21	76.86 ± 4.15	255.15 ± 6.32	3.33 ± 0.12	26.24 ± 1.34	217.64 ± 3.54	8.36 ± 0.43	24.58 ± 2.62
22	60.45 ± 4.75	484.76 ± 5.76	8.11 ± 0.51	25.31 ± 1.07	496.11 ± 8.20	19.7 ±0.83	26.24 ± 1.97
23	33.36 ± 2.05	391.30 ± 5.63	11.8 ± 0.68	9.28 ± 0.46	385.39 ± 9.63	41.77 ± 1.92	36.45 ± 1.78
24	28.86 ± 1.14	395.66 ± 7.11	13.73 ± 0.30	7.17 ± 0.50	390.90 ± 4.35	55.14 ± 3.34	39.02 ± 2.49
25	145.82 ± 8.81	448.40 ± 12.44	3.1 ± 0.2	111.77 ± 6.90	429.20 ± 14.30	3.86 ± 0.14	12.19 ± 0.84
26	64.45 ± 5.42	232.91 ± 24.97	3.61 ± 0.22	72.09 ± 5.98	190.70 ± 10.33	2.71 ± 0.34	23.08 ± 1.34
27	106.31 ± 8.78	368.27 ± 19.37	3.49 ± 0.12	65.56 ± 3.54	352.85 ± 10.75	5.41 ± 0.17	24.39 ± 1.08
28	162.00 ± 4.92	516.72 ± 26.11	3.21 ± 0.25	101.20 ± 9.30	476.85 ± 33.73	4.76 ± 0.29	18.29 ± 1.22
29	103.27 ± 8.03	511.69 ± 40.92	4.95 ± 0.08	59.56 ± 13.88	491.14 ± 42.23	8.3 ± 0.74	25.84 ± 2.62
30	68.32 ± 8.57	580.09 ± 13.67	8.95 ± 1.24	53.27 ± 5.65	565.28 ± 21.45	10.96 ± 1.23	24.23 ± 1.34
31	93.62 ± 5.52	316.92 ± 28.78	3.37 ± 0.14	52.92 ± 3.22	343.36 ± 17.38	6.52 ± 0.36	27.14 ± 2.25
32	67.45 ± 6.94	373.05 ± 19.48	5.66 ± 0.53	33.10 ± 2.79	404.26 ± 19.50	12.38 ± 0.83	27.31 ± 2.28
33	56.16 ± 4.24	528.76 ± 53.53	9.36 ± 0.38	19.60 ± 1.36	497.88 ± 26.12	25.87 ± 2.69	34.28 ± 2.45
34	43.27 ± 1.44	374.27 ± 6.23	8.56 ± 0.19	8.54 ± 0.67	356.84 ± 5.39	42.17 ± 2.60	39.42 ± 1.85
35	20.13 ± 0.91	305.46 ± 2.38	15.2 ± 0.62	4.25 ± 0.26	346.20 ± 6.02	82.24 ± 3.77	38.83 ± 2.11

1 = 50% Inhibitory concentration (IC_{50}); 2 = IC_{50} against RAW264.7 cells; 3 = Therapeutic index (TI); 4 = 50% Effective concentration (EC_{50}); 5 = EC_{50} against mouse peritoneal macrophages.

Table 3
Antipromastigote and Antiamastigote activities against *L. donovani*, TI values and nitric oxide generation of compound 36 to 38.

Comp.	Promastigote			Amastigote			NO generation (μM)
	[1]IC_{50} (μM)	[2]Cytotoxicity IC_{50} (μM)	[3]TI	[4]EC_{50} (μM)	[5]Cytotoxicity EC_{50} (μM)	[3]TI	
36	97.35 ± 3.90	278.43 ± 6.31	2.87 ± 0.08	72.90 ± 3.18	325.54 ± 5.28	4.49 ± 0.25	21.87 ± 1.37
37	89.01 ± 5.92	261.34 ± 5.45	2.96 ± 0.16	66.32 ± 2.87	306.74 ± 5.41	4.66 ± 0.29	21.55 ± 1.22
38	52.97 ± 1.57	450.56 ± 5.82	8.6 ± 0.09	21.55 ± 1.23	471.12 ± 10.10	22.06 ± 1.27	28.78 ± 1.28

explicitly showed the importance of the spacer length for the betterment of their activity. Now for the aliphatic substituents (**17** and **25** to **35**), the EC_{50} values were found to improved with the increase of hydrophobicity (carbon chain length) with two exceptions (**25** and **28**). The best activity was found for comp.**35** (4.25 ± 0.26 μM) in this set of molecules with the TI value of 82.24 ± 3.77. The nitric oxide generation by comp.**35** (38.83 ± 2.11 μM) was found to be comparable with Miltefosine (26.01 ± 2.85 μM).

The third set of molecules **36**, **37** and **38** showed moderate activity against amastigotes. Out of them, comp.**38** with an additional primary amino group showed the best EC_{50} value (21.55 ± 1.23 μM), with a corresponding TI value of 22.06 ± 1.27.

The dose ratio for **22**, **23**, **24** and **38** in case of the direct killing (against promastigote) versus macrophage mediated killing (against amastigote) was found to lies in the range of 2.3–4. A careful comparison of the TI values for molecule **22**, **23**, **24** and **38** with others suggested that this improvement is because of the concomitant decrease of the effective dose with the decreased cytotoxicity against both promastigote and amastigote. However, a desired TI value (>40) was achieved with only **23** (TI 41.77 ± 1.92), **24** (TI 55.14 ± 3.34), **34** (42.17 ± 2.60) and **35** (82.24 ± 3.77). For both of these compounds lower EC_{50} values showed that an increase of the substituent length has a decisive role in the immunomodulatory activity. Similar to the Miltefosine, introduction of the C16 carbon chain with eugenol via ether (**15**) and ester (**34**) linkage enhanced the TI value for **34** (42.17 ± 2.60) against amastigote, although for the ether linkage (**15**) the corresponding value was reached only upto 14.47 ± 0.91. The selective increase of TI value against amastigotes (for **34**) proved that increased hydrophobicity plays a decisive role for the immunomodulatory activity.

All the synthesized molecules except **5**, **6, 18** and **26** showed better inhibitory activity against amastigote compared to their corresponding IC_{50} value against promastigote. This clearly suggested that the synthesized molecules preferentially clear the parasitic burden by immunomodulation rather than the direct killing of the *L. donovani*.

Comparison of the antileishmanial activity between three set of molecules showed that the activity is mostly dependent of the hydrophobicity of the molecules except for **23** and **24**. For the similar aliphatic substitutions (where $R_1 = R_2$), we observed an improvement in their antileishmanial (IC_{50} and EC_{50}) activity for set I and set II molecules with few exceptions. However, for the set III molecules, containing an amino moiety did not show any appreciable activity on *L. donovani*, although it is better than comp.**1** (EC_{50} = 173.26 ± 7.37 μM) and **2** (EC_{50} = 203.28 ± 9.03 μM).

2.3. Correlation between log P and therapeutic index

Log P (partition coefficient) measurements is a useful parameter in understanding the behavior of drug molecules. Therefore, to find out differential behaviour of the synthesized molecules we calculated their log P values and compared it with our experimental parameters. From the first set of molecules (**1** to **16**), the higher TI values were observed from **9** to **16,** with the log P value in a range of 4.86–9.23 (Fig. 1). The maximum TI, 15.94 ± 1.79 was recorded for comp.**16** with a log P value of 9.23. Whereas, other molecules with log P values between 2.12 and 4.35 did not showed appreciable TI

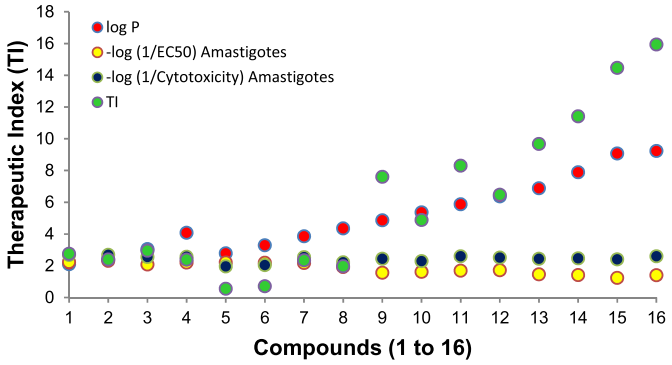


Fig. 1. Correlation between log P, TI, EC_{50} against amastigotes and EC_{50} for cytotoxicity for **1** to **16.**

value. Thus the improvement of the TI values for this set of molecules can be well corroborated with the increased hydrophobicity of the molecules.

For the second set of molecules (**17** to **35**), we altered the log P value from 1.90 to 9.00, which leads to the emergence of four lead molecules **23**, **24**, **34** and **35** with significant TI value (>40). However, 35 (TI = 82.24 ± 3.77) was the best lead molecule among the others. The correlation between the log P and TI revealed that the most favoured log P values were 3.50 (comp.**23**) and 3.38 (comp.**24**) for molecules having aromatic substitution. Whereas, for aliphatic substitution a concomitant exponential increase in the TI value was observed with the increase of log P value by virtue of the chain length elongation (Fig. 2). A careful observation of the EC_{50} values and nitric oxide generation for comp. **23**, **24**, **34** and **35**, indicated that the activity against amastigotes got increased with the increase of log P and at the same time nitric oxide generation was also increased to a substantial level.

For the third set of molecules **36** to **38** the best TI value was observed for comp. **38** (TI = 22.06 ± 1.27) with a log P value of 2.51 (Fig. 3). However, for the known drugs Miltefosine and Amp-B the calculated TI values were 6.34 ± 0.45 and 7.78 ± 0.59 respectively, which was solely because of their higher toxicity.

Unlike comp. **23** and **24**, for the other molecules **6**, **7** and **27** a significant increase of the TI value was not observed despite having the comparable log P values. This provided an indication that may be their mode of interaction with the macrophage is different. In general molecule with high log P value (>7) is considered to be toxic because of their unspecific interaction with the cellular protein. However, the high TI value for **35** (log P = 9.00) may be arise due to its interaction with the receptors embedded on the

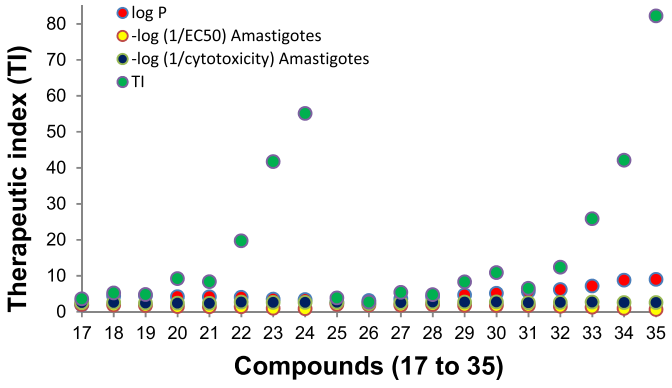


Fig. 2. Correlation between log P, TI, EC_{50} against amastigotes and EC_{50} for cytotoxicity for comp. **17** to **35**.

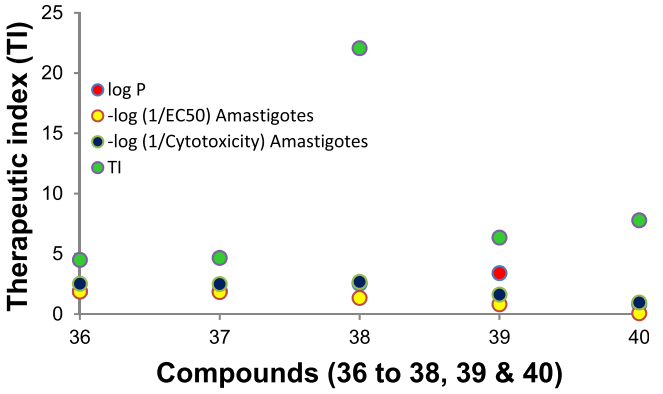


Fig. 3. Correlation between log P, TI, EC_{50} against amastigotes and EC_{50} for cytotoxicity for comp. **36** to **38**. Miltefosine and Amphotericin b are marked as 39 and 40, respectively.

membrane rather than interaction with the cellular proteins. Concurrently, the lower TI value of **16** (log P = 9.23) compared to **35** (log P = 9.00) can be attributed because of their different physicochemical properties. However, this observation revealed the preference of the ester linkage over the ether linkage.

2.4. Cellular uptake of eugenol and its derivatives

Incorporation of the oleyl group was found to be a decisive factor to obtain high TI value. However, incorporation of the same aliphatic chain did not show such elevation of TI value for comp.**16** as compared to comp.**35**. Therefore, we were quite curious to know about its possible reason. There are two possible factors responsible for this observation. In the first case, **35** can act as a prodrug which mitigate the transport of the eugenol inside the macrophage and thus elevate the TI value unlike comp.**16** or **1**. In the second case, it can simply increase the macrophage intake (inside the cell or attachment with the surface receptor) by virtue of its chemical structure. Therefore, to probe this question we tested the stability of **16**, **24** and **35** along with Eugenol (1) in an HPLC-based assay. To determine the stability and permeability of the above mentioned compounds, we treated macrophage in culture medium (RPMI-1640) for 3 h at 37 °C with **1** (10 μg/ml), **16** (10 μg/ml), **24** (10 μg/ml) and **35** (10 μg/ml) separately. The residual compound present in the supernatant and in cell lysate were analyzed by HPLC. Presence of the individual species as a major component in media and with the cell (for 3 h) unambiguously proved that they are not at all a prodrug (SI:S4; Fig S3 to S10). Then we determined the permeability of individual molecules to address the second possibility using the equation (1).

$$\textit{Cell permiability} = \frac{A_{CL}}{(A_S + A_{CL})} \quad (1)$$

where, A_{CL} and A_S are the area under curve for the respective molecules.

The cell permeability values for comp. **1**, **16**, **24** and **35** were

Table 4
Cell permeability of comp. **1**, **16**, **24**, and **35**.

Comp.	R_t (min)	A_S	A_{CL}	$A_{CL}/(A_S + A_{CL})$
1	3.133	9140911	2512550	0.21
16	3.147	626918	793072	0.56
24	3.867	2180634	5815021	0.73
35	3.173	1115249	1803673	0.62

found 0.21, 0.56, 0.73 and 0.62 respectively (Table 4). This experimental observation unequivocally proved that the better antileishmanial activity with elevation of TI value is because of their improved interaction with the macrophage, which fundamentally depends on the physico-chemical nature of a molecule.

2.5. Binding of comp. **35** with macrophages

To find out the lower toxicity of comp.**35** despite the high log P, we checked its attachment with RAW264.7 macrophage using fluorescence microscopy. A fluorophoric moiety was attached with **35** via carbamate linkage (SI1) in order to get fluorescence signal under fluorescence microscope. Accumulation of **35** in the images (Fig. 4A) indicated that **35** interact with the plasma membrane of the macrophages.

2.6. Effect of comp. **35** on NO generation and arginase 1 activity

The clearance of intracellular amastigotes within the macrophages is solely mediated by NO generation [14]. Thus, we studied comp.**35** mediated NO generation in BALB/c derived peritoneal macrophages. Comp.**35** induced the NO generation in a significant dose dependent manner ($p < 0.01$) at 1 μg/ml (2.33 μM) (~3 folds increase), 2.5 μg/ml (5.82 μM) (~7 folds increase) and 5 μg/ml (11.65 μM) (~8 folds increase) compared to untreated infected macrophages (Fig. 5A). Concomitantly, *iNOS2* mRNA expression levels was found to increase by ~8 folds in *L. donovani* infected macrophages compared to the control (Fig. 5B–C). Therefore, it signified that the dose dependent increase of NO generation was obligated by the *iNOS2* activation in macrophages [15,16].

Increase in arginase 1 activity serves as a signature for the manifestation of leishmanial infection inside the macrophages [17,18]. Therefore, we studied the effect of **35** on the arginase 1 activity in infected peritoneal macrophages. The spectrophotometric facts, as well as, RT-PCR data showed that arginase 1 activity and its corresponding mRNA expression were decreased by ~4 folds significantly ($p < 0.01$) by **35** no molecule compared to untreated infected macrophages (Fig. 5D–F). From the results, it was evidenced that **35** not only increases the intracellular leishmanicidal NO generation, but also decreases the disease progression factor, arginase 1. The parasites increase the arginase 1 activity in infected macrophages, which result in decreased availability of L-arginine for the macrophages to produce NO free radicals through *iNOS2* [15–18]. Comp.**35** mediated decrease in arginase 1 activity might have increased the availability of L-arginine for the macrophages that activated *iNOS2* to induce more NO generation in *L. donovani* infected macrophages.

2.7. Comp.35 treatment enhanced the IL-12 and reduced the IL-10 production in L. donovani infected peritoneal macrophages

Proinflammatory cytokine, IL-12, is known to play a decisive role in the induction of NO generation through *iNOS2* activation to cure visceral leishmaniasis [19,20]. Whereas, IL-10 is an anti-inflammatory cytokine counter regulates the production of IL-12, there by affect the NO generation and promotes disease progression [21]. Since, NO generation and *iNOS2* expression were significantly elevated in **35** treated parasitized macrophages. Hence, we have also evaluated IL-12 and IL-10 release in infected peritoneal macrophages upon comp.**35** treatment. Our results depict that **35** efficiently increased IL-12 level (~5 folds) (Fig. 6A) and suppressed IL-10 level (~3 folds) (Fig. 6C) in *L. donovani* infected macrophages. Comp.**35**-induced amendment of IL-12 and IL-10 release were further confirmed by studying their mRNA expression by conventional RT-PCR (Fig. 6B–C and 6E-F). It is well known that IL-12 involves in the activation and differentiation of immune cells, as well as, important for the parasite clearance inside the macrophages. However, it generally gets suppressed to facilitate the 'silent entry' of the parasite into host macrophages [22]. Conversely, IL-10 is essential for parasite survival and diseases progression, and it gets induced in *L. donovani* infected macrophages [21,23]. The comp.35 induced rectification of IL-12 and IL-10 level may be correlated with the increased NO generation to endorse the *L. donovani* killing inside the macrophages. This indicates the immunomodulatory activity of 35 to induce host protective immune response through the activation proinflammatory cytokines.

2.8. Comp. 35 reduced the hepatic and splenic parasite burden in L. donovani infected BALB/c mice

The *in vivo* efficacy of **35** was tested in BALB/c mouse model to elucidate the antileishmanial and the immunomodulatory potential of **35** in restricting *L. donovani* induced pathogenesis. As, *L. donovani* induced visceral leishmaniasis manifests as the enlargement of spleen and liver due to the accumulation of parasite [15,20]. Hence, we selectively studied the hepatic and splenic parasite burden in infected BALB/c mouse treated with **35**. The results indicate that comp.**35** was able to cause 81% and 92% of hepatic parasite clearance (Fig. 7A) and 72% and 87% of splenic parasite clearance (Fig. 7B) in the infected BALB/c mouse treated with 10 mg/kg body weight (B.W.) and 25 mg/kg B.W. respectively, after 28 days of treatment following two weeks of post infection. Hence, comp.**35** not only increased the parasite killing *in vitro*, but also equally effective *in vivo* experimental model of visceral leishmaniasis.

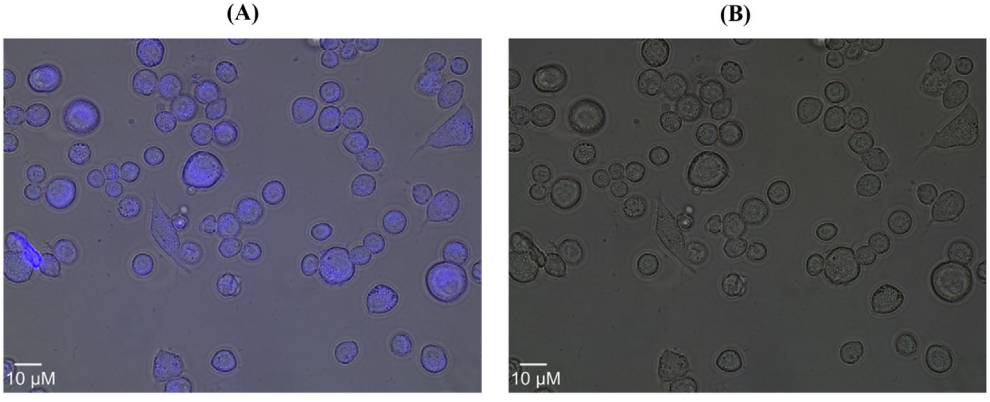


Fig. 4. Fluorescence image (A) and its DIC image (B) of compound **35** treated RAW264.7 macrophages.

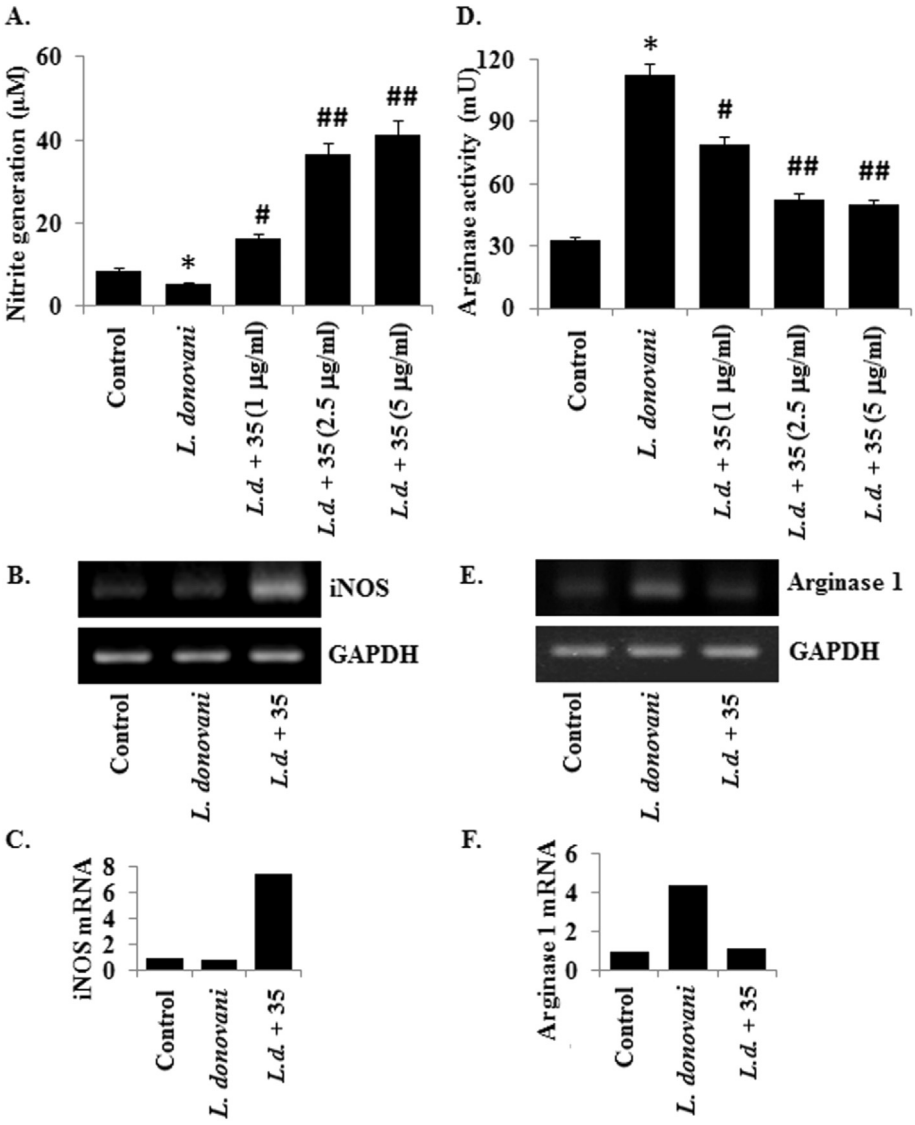


Fig. 5. Effect of comp. 35 treatment on NO generation and the arginase 1 activity in *L. donovani* infected BALB/c derived peritoneal macrophages. (A) BALB/c derived peritoneal macrophages were cultured and infected by *L. donovani* promastigotes as described in materials and methods section. Infected macrophages were treated with comp. **35** with indicated concentrations [1 μg/ml (2.33 μM); 2.5 μg/ml (5.82 μM); 5 μg/ml (11.65 μM)]. After 48 h of incubation, nitrite generation was estimated from cell-free supernatants as described in methods section. Data for nitrite generation were expressed as mean ± S.E.M. from triplicate experiments yielding similar type of results. (D) Peritoneal macrophages were cultured, infected with *L. donovani* promastigotes followed by treatment with indicated concentrations of comp. **35** as described earlier. After 48 h treatment, cell lysates were used for the arginase 1 activity by spectrophotometric method. The bar represents the mean ± S.E.M. of arginase 1 activity from three independent experiments that yielded similar type of results. (B & E), Untreated uninfected, untreated infected and **35** [2.5 μg/ml (5.82 μM)] treated infected peritoneal macrophages were incubated for 6 h, after which the cells were collected in TRIZOL. Then RNA was extracted, and the mRNA expression was studied by conventional RT-PCR using *iNOS2* and *arginase 1*-specific primers, as described in materials and methods section. The data represent the best of triplicate experiments with similar type of results. (C & F) The density units shown in the bar diagram are arbitrary units obtained from the densitometry analysis using the Quantity-One software, Bio-Rad. The data represent from three independent experiments that yielded similar type of results. $^{*}p < 0.05$, compared with uninfected control macrophages; $\#p < 0.05$, $\#\#p < 0.01$ compared with infected macrophages.

2.9. Comp.35 treatment increased the NO generation and Th1 cytokines response in L. donovani infected BALB/c mice

The NO generation, arginase 1 activity and cytokine profiles, studied from splenocytes, were in well accordance with the *in vitro* studies. These experimental data unambiguously prove the potential immunotherapeutic activity of comp.**35** against *L. donovani* persuaded pathogenesis. Our results unequivocally suggest that the protective response caused by comp.**35** against visceral leishmaniasis is due to the strong induction of NO generation (~8 folds increase) through *iNOS2* expression (~8 folds increase) coupled with decreased arginase 1 activity (~4 folds; protein/mRNA) in splenocytes (Fig. 8A–B, F-G). Results of cytokine release and mRNA expression level revealed the significant ($p < 0.01$) increase in IL-12 (Fig. 8C, H), IFN-γ (Fig. 8D, I) and decrease in IL-10 (Fig. 8E, J), which provides the indication of skewed Th2 cytokine response towards Th1 cytokine response in *L. donovani* infected mice treated with **35** [9,24]. Hence the *in vitro* and *in vivo* results indicate the host protective immunomodulatory role of 35 in treating visceral leishmaniasis through NO generation and Th1 cytokine response.

3. Conclusion

Among the 36 different eugenol derivatives **23**, **24**, **34** and **35** showed an improved TI value (>40) compared to the available drugs Miltefosine (TI 6.34 ± 0.45) and Amphotericin B (TI

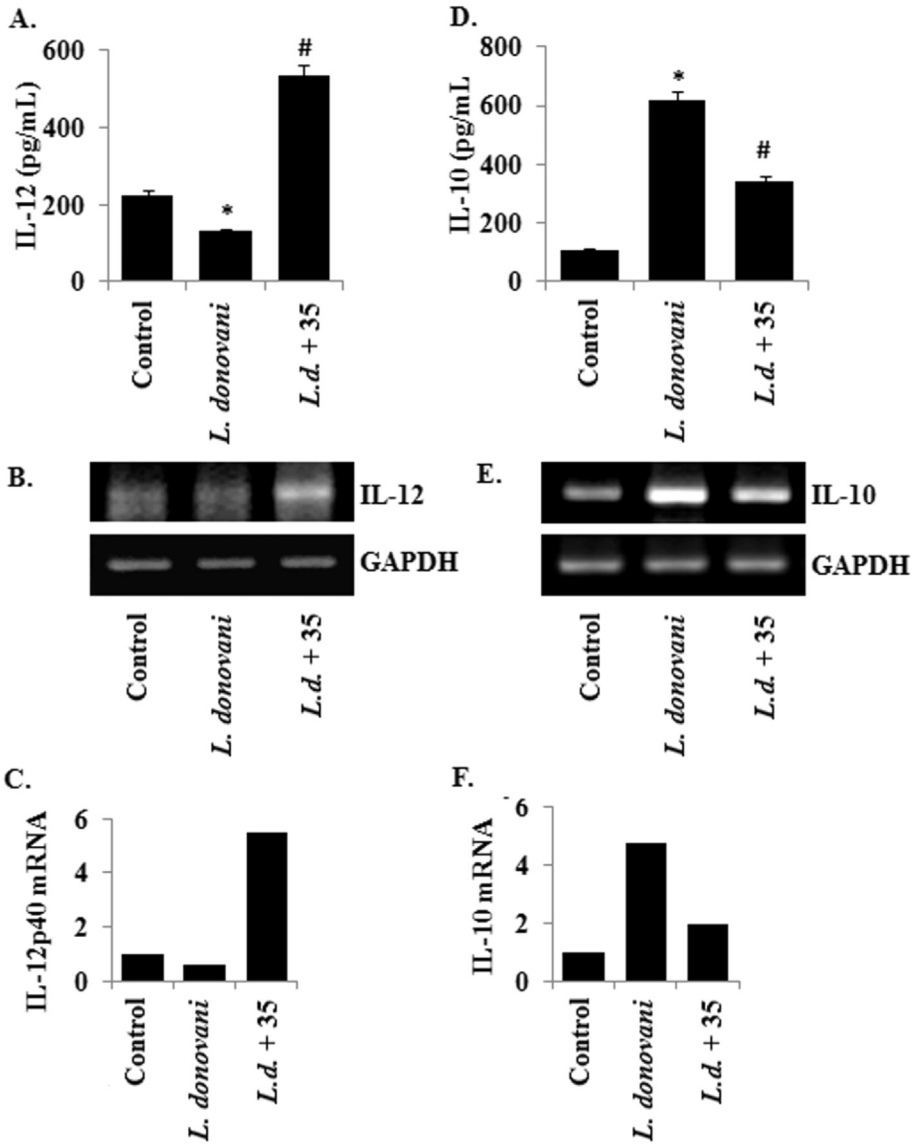


Fig. 6. Effect of comp. 35 treatment on IL-12 and IL-10 mRNA expression and release in *L. donovani* infected peritoneal macrophages. (A & D), BALB/c derived peritoneal macrophages were cultured, infected with *L. donovani* promastigotes followed by treatment with **35** [2.5 μg/ml (5.82μM)] as described earlier. After 24 hrs the cell-free supernatants were collected and subjected to sandwich ELISA to detect (A) IL-12 (pg/ml), (D) IL-10 (pg/ml) production by macrophages. Results were expressed as mean ± S.E.M. from three replicate experiments yielding similar type of results. $^{*}p < 0.05$, significant differences compared with uninfected control macrophages; and $\#p < 0.01$, significant differences compared with infected macrophage. (B & E), A separate set of control, infected and infected-treated [**35**, 2.5 μg/ml (5.82 μM)] peritoneal macrophages were collected in TRIZOL after 6 h. The RNA was extracted, and the mRNA expression was studied by conventional RT-PCR using *il-12* (B), *il-10* (E) specific primers, as described in materials and methods section. The data represent the best of triplicate experiments with similar type results. (C & F), The density units (C, *il-12*; F, *il-10*) shown in the bar diagram are arbitrary units obtained from the densitometry analysis using the Quantity-One software, Bio-Rad.

7.78 ± 0.59). The highest TI value against the amastigotes (82.24 ± 3.77) was obtained for the comp.**35**, whereas its IC_{50} (considered for promastigotes) and EC_{50} (considered for amastigotes) values were 20.13 ± 0.91 μM and 4.25 ± 0.26 μM, respectively. The corresponding TI value against the promastigote was 15.2 ± 0.62. Thus, the 5.41 folds increase of TI value and 4.74 folds lower EC_{50} value against the amastigote compared to promastigote proved the potent immunomodulatory activity of comp.**35**. This finding was also supported by the NO generation in treated infected macrophages. It was observed that with a high log P value, comp.**35** preferentially binds with the membrane bound receptors and imparts its antileishmanial activity. The strong proinflammatory response (IL-12, IFNγ) was found to counter regulate the anti-inflammatory response (IL-10) in presence of comp.**35** in infected BALB/c mouse model. This is in consequence increased the iNOS2 expression by 12 folds compared to the infected mice which further may due to the decrease of arginase1 activity and its expression. All together, this study shows the potential application of eugenol derivative **35** as a chemotherapeutic agent against visceral leishmaniasis. Finally, this work indicates how a simple structural alteration can impart potent immunomodulatory activity to a molecule with moderate activity, compared to the available anti-leishmanial drugs.

4. Experimental

4.1. Reagents and instrumentation

All the chemicals, standard drugs were purchased from Sigma–Aldrich, Alfa Aesar and Merck Chemicals. Column

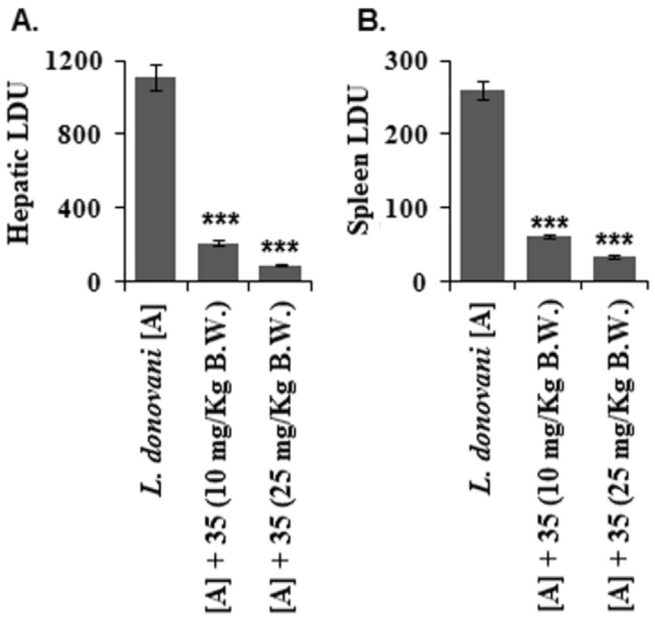


Fig. 7. Hepatic and splenic parasite burden in *L. donovani*-infected BALB/c mice treated with comp. 35. Two weeks post infected mice were treated by **35** with indicated doses *i.v.* for another 28 days. There after mice were sacrificed and level of parasite burden was determined in liver (A) and spleen (B) by the stamp smear method, subsequently expressing as Leishman-Donovan Unit (LDU). Results were expressed as mean ± S.E.M. from four mice per group. $^{***}p < 0.001$, significant differences compared with infected mice.

chromatographic separations were performed using silica gel (100–200 mesh). Solvents were dried and distilled following standard procedures. TLC was carried out on pre-coated plates (Merck silica gel 60, f_{254}), and the spots were visualized with UV light or by charring the plates dipped in 10% PMA solution in methanol or 5% H_2SO_4/vanillin/EtOH. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker NMR spectrometer (δ scale). Mass spectra had been recorded using Waters Mass Spectrometer (model XevoG2QTof). The log P values were calculated using Molinspiration Cheminformatics software (www.molinspiration.com) Griess reagent and sequence specific Oligos were purchased from Sigma-Aldrich. RPMI1640, fetal bovine serums (FBS) were obtained from Gibco and ELISA kits of IL-10, IL-12 and IFN-γ were purchased from R&D systems. cDNA synthesis kit and RT-PCR chemicals were obtained from Fermentas. Real-time PCR chemicals were procured from TAKARA Bio. Plastic wares were purchased from Genetix and Thermo.

4.2. Synthesis of the eugenol derivatives

4.2.1. Formation of the ether linkage: general method

Method A1: Eugenol was dissolved in dry THF and stirred at room temperature under nitrogen atmosphere. To the stirred solution triphenylphospine (TPP) and corresponding alcohol were added and the stirring was continued for 15 min at room temperature. Thereafter, diisopropylazodicarboxylate (DIAD) was added drop wise to the reaction mixture, and stirred at room temperature for another 8–12 h. After completion of reaction (checked by TLC) volatiles were evaporated and extracted with ethylacetate. The combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The compound thus obtained was purified by silica-gel column chromatography using ethyl acetate and hexanes as eluent.

Method B1: To a stirred solution of eugenol in DMF at 0 °C, NaH was added and stirred for 10 min. Thereafter, measured amount of alkyl/alkenyl halides were added and the reaction mixture was stirred for another 2 h at room temperature. After completion of the reaction it was quenched with water and diluted with EtOAc. The organic portion was then washed with water and brine solution. The organic portion was the dried over Na_2SO_4 and concentrated under reduced pressure. The compound thus obtained was purified by silica-gel column chromatography using ethyl acetate and hexanes as eluent.

4.2.2. Formation of the ester linkage: general method

Method A2: Eugenol was dissolved in chloroform and cooled to 0–5 °C, to that pyridine was added followed by the addition of respective acid anhydride. The reaction mixture was then stirred at room temperature for 6 h. After completion of reaction (checked by TLC) it was diluted with chloroform and washed with 5% aqueous HCl, water and brine. Combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The compound thus obtained was purified by silica-gel column chromatography using ethyl acetate and hexanes as eluent.

Method B2: Eugenol was dissolved in DMF. To that 4-(dimethylamino) pyridine (DMAP) and respective acid were added and stirred at room temperature. After 15 min *N*, *N*-dicyclohexylcarbodiimide (DCC) was added dropwise and stirred at room temperature for 10 h. After completion of reaction (checked by TLC), reaction mixture was diluted with water and extracted with Ethyl acetate (3 × 30 ml). Combined organic layer was then washed with saturated $NaHCO_3$, water and then with brine solution. Combined organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The compound thus obtained was purified by silica-gel column chromatography using ethyl acetate and hexanes.

Method C2: Carboxylic acid, EDCI.HCl and DMAP were suspended in 3 ml of THF and stirred for 20 min at room temperature. Then eugenol was added to the reaction mixture and allowed to stir for 4 h at room temperature. After the completion of reaction (checked by TLC), all the volatile compounds were evaporated in vacuo. The crude was partitioned between ethyl acetate (3 × 10 ml) and 10% sodium bicarbonate. The combined organic layer was washed with water followed by brine and dried using Na_2SO_4. Then, the compound was purified by silica gel chromatography.

*4.2.2.1. 4-Allyl-1-allyloxy-2-methoxy-benzene (**3**).* Method A1: Amount of Eugenol (0.1 g; 0.6 mmol), TPP (0.192 g; 0.73 mmol), DIAD (0.148 g; 0.73 mmol), allyl alcohol (0.035 g; 0.6 mmol) and THF (4 ml). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 71.2%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.32 (d, J = 6.6 Hz, 2H), 3.86 (s, 3H), 4.59 (d, J = 5.4 Hz, 2H), 5.05–5.11 (m, 2H), 5.28 (d, J = 13.5 Hz, 1H), 5.39 (dd, J = 1.2, 17.4 Hz, 1H), 5.89–6.15 (m, 2H), 6.68–6.72 (m, 2Ar-H), 6.81 (d, J = 8.1 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 39.8, 55.8, 69.9, 112.2, 113.5, 115.6, 117.7, 120.3, 133.0, 133.5, 137.6, 146.2, 149.3. HRMS (ESI+): m/z calcd for $C_{13}H_{17}O_2$ $[M+H]^+$: 205.1229; found: 205.1226.

*4.2.2.2. 4-Allyl-2-methoxy-1-(3-methyl-but-2-enyloxy)-benzene (**4**).* Method A1: Amount of Eugenol (0.1 g; 0.6 mmol), TPP (0.192 g; 0.73 mmol), DIAD (0.148 g; 0.73 mmol), prenol (0.052 g; 0.6 mmol) and THF (4 ml). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 74.8%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.74 (d, J = 12.6 Hz, 6H), 3.33 (d, J = 6.9 Hz, 2H), 3.85 (s, 3H), 4.56 (d, J = 6.6 Hz, 2H), 5.04–5.11 (m, 2H), 5.52 (t, J = 6.6 Hz, 1H), 5.90–6.03 (m, 1H), 6.69–6.71 (m, 2Ar-H), 6.81 (d, J = 8.7 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 18.2, 25.8, 39.8, 55.8, 65.9, 112.1, 113.2, 115.6, 120.2, 120.3, 132.7,

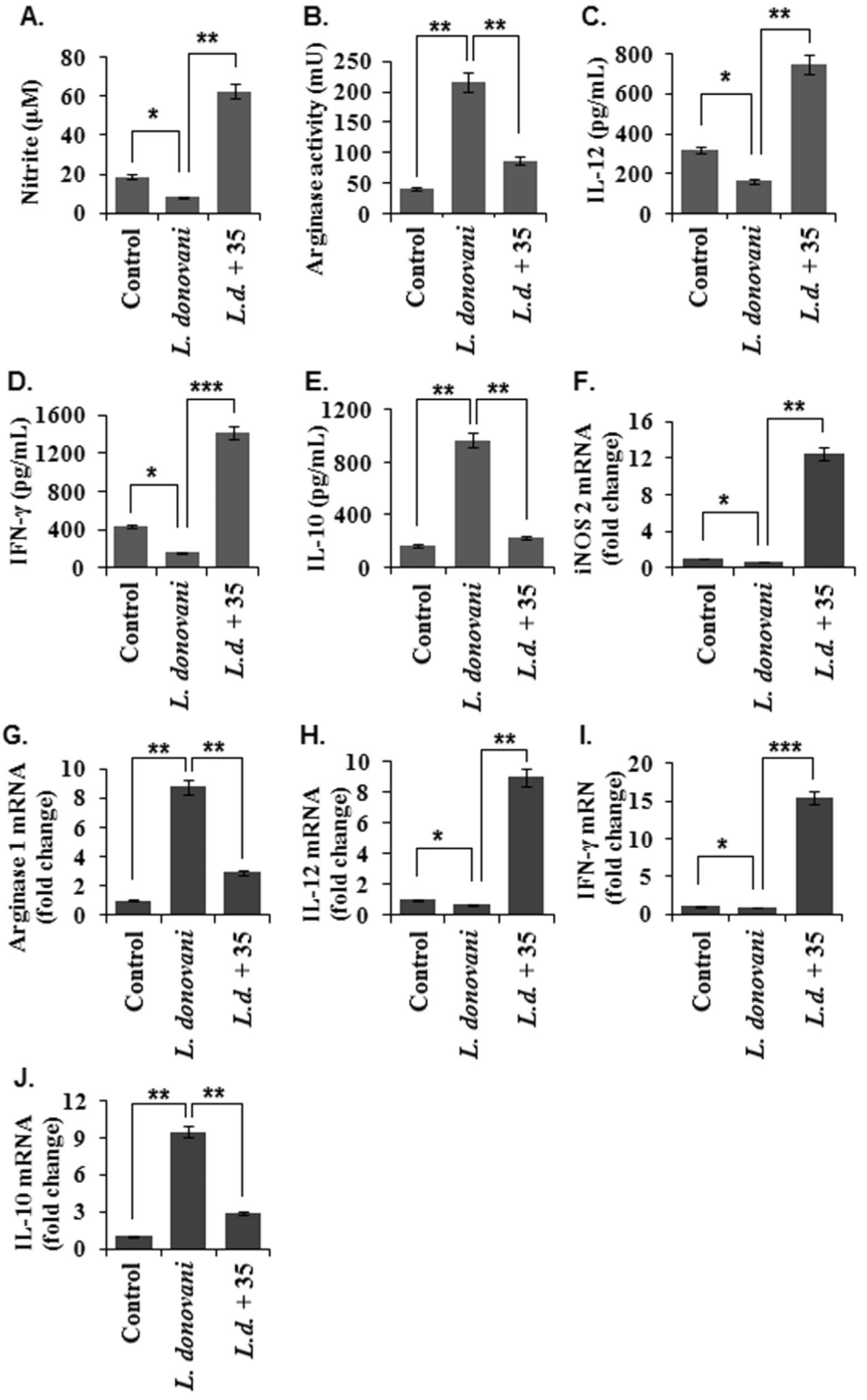


Fig. 8. Effect of comp. 35 treatment on NO generation and Th1 cytokines response in *L. donovani* infected BALB/c mice. (A–E) Isolated splenocytes from indicated groups were seeded aseptically (2×10^6) and stimulated with soluble leishaminal antigen (5 μg/ml) for 48 h. Level of nitrites, cytokines were estimated from cell free supernatant and arginase 1 activity was studied from cell lysates as described in the methods section. (F–J), Real time polymerase chain reaction assay was done from separate sets of splenocytes collected in Trizol to determine the mRNA expression of *iNOS2*, *arginase 1*, *il-12*, *ifn-γ* and *il-12* as described in the methods section. The experimental data represented here as the mean ± S.E.M. from 4 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significant differences compared with respective control group.

137.4, 137.7, 146.6, 149.4. HRMS (ESI+): *m/z* calcd for $C_{15}H_{21}O_2$ $[M+H]^+$: 233.1542; found: 233.1538.

*4.2.2.3. 4-Allyl-1-ethoxy-2-methoxy-benzene (**5**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.016 g; 0.69 mmol) and 1-Bromoethane (0.086 ml; 1.15 mmol). Yield: 70.7%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.45 (t, J = 7.2 Hz, 3H), 3.33 (d, J = 6.9 Hz, 2H), 3.86 (s, 3H), 4.08 (q, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.90–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.9, 39.8, 55.9, 64.4, 112.1, 112.9, 115.6, 120.4, 132.7, 137.7, 146.6, 149.2. HRMS (ESI+): *m/z* calcd for $C_{12}H_{17}O_2$ $[M+H]^+$: 193.1229; found: 193.1227.

*4.2.2.4. 4-Allyl-2-methoxy-1-propoxy-benzene (**6**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol) and 1-Bromopropane (0.085 g; 0.69 mmol). Yield: 56.9%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.03 (t, J = 7.5 Hz, 3H), 1.85 (sextet, J = 7.2 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.95 (t, J = 6.6 Hz, 2H), 5.04–5.11 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.83 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 10.5, 22.5, 39.8, 56.0, 70.7, 112.4, 113.2, 115.6, 120.5, 132.7, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{13}H_{19}O_2$ $[M+H]^+$: 207.1385; found: 207.1386.

*4.2.2.5. 4-Allyl-1-butoxy-2-methoxy-benzene (**7**).* Method A1: Amount of eugenol (0.1 g; 0.6 mmol), triphenylphospine (0.192 g; 0.73 mmol), *n*-Butanol (0.044 g; 0.6 mmol) and DIAD (0.148 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 79.8%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.89 (t, J = 8.4 Hz, 3H), 1.35–1.47 (m, 2H), 1.69–1.79 (m, 2H), 3.26 (d, J = 6.6 Hz, 2H), 3.78 (s, 3H), 3.92 (t, J = 6.9 Hz, 2H), 4.97–5.04 (m, 2H), 5.82–5.95 (m, 1H), 6.62–6.64 (m, 2Ar-H), 6.75 (d, J = 8.7 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 13.8, 19.2, 31.3, 39.8, 55.9, 68.8, 112.3, 113.1, 115.5, 120.4, 132.6, 137.7, 146.9, 149.3. HRMS (ESI+): *m/z* calcd for $C_{14}H_{21}O_2$ $[M+H]^+$: 221.1542; found: 221.1540.

*4.2.2.6. 4-Allyl-2-methoxy-1-pentyloxy-benzene (**8**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol) and 1-Bromopentane (0.104 g; 0.69 mmol). Yield: 60.6%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.92 (t, J = 6.9 Hz, 3H), 1.37–1.49 (m, 4H), 1.84 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.0, 22.5, 28.1, 29.0, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{15}H_{23}O_2$ $[M+H]^+$: 235.1698; found: 235.1693.

*4.2.2.7. 4-Allyl-1-hexyloxy-2-methoxy-benzene (**9**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol) and 1-Bromohexane (0.16 ml; 1.146 mmol). Yield: 56.9%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.90 (t, J = 6.9 Hz, 3H), 1.25–1.50 (m, 6H), 1.83 (quintet, J = 7.2 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.99 (t, J = 7.2 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.6, 25.7, 29.2, 31.6, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{16}H_{25}O_2$ $[M+H]^+$: 249.1855; found: 249.1859.

*4.2.2.8. 4-Allyl-1-heptyloxy-2-methoxy-benzene (**10**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol) and 1-Bromoheptane (0.18 ml; 1.146 mmol). Yield: 76.6%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.30–1.47 (m, 8H), 1.83 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 3.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.6, 26.0, 29.1, 29.3, 31.8, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{17}H_{27}O_2$ $[M+H]^+$: 263.2011; found: 263.2008.

*4.2.2.9. 4-Allyl-2-methoxy-1-octyloxy-benzene (**11**).* Method B1: Amount of Eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol) and 1-Bromooctane (0.133 ml; 0.69 mmol). Yield: 65.7%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.28–1.47 (m, 10H), 1.83 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 29.2, 29.4, 31.8, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{18}H_{29}O_2$ $[M+H]^+$: 277.2168; found: 277.2170.

*4.2.2.10. 4-Allyl-2-methoxy-1-nonyloxy-benzene (**12**).* Method B1: Amount of eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol), 1-Bromononane (0.142 g; 0.6 mmol) and DIAD (0.148 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 80.1%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.27–1.46 (m, 12H), 1.83 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 29.3, 29.3, 29.4, 29.5, 31.9, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{19}H_{31}O_2$ $[M+H]^+$: 291.2324; found: 291.2320.

*4.2.2.11. 4-Allyl-1-decyloxy-2-methoxy-benzene (**13**).* Method B1: Amount of eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.73 mmol), 1-Bromodecane (0.134 ml; 0.69 mmol) and DIAD (0.148 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 74.1%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.46 (m, 14H), 1.82 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 7.2 Hz, 2H), 5.04–5.11 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 29.3, 29.3, 29.4, 29.6, 31.9, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{20}H_{33}O_2$ $[M+H]^+$: 305.2418; found: 305.2411.

*4.2.2.12. 4-Allyl-1-dodecyloxy-2-methoxy-benzene (**14**).* Method B1: Amount of eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol), 1-Bromododecane (0.165 ml; 0.69 mmol) and DIAD (0.148 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 49.5%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.46 (m, 18H), 1.82 (quintet, J = 6.9 Hz, 2H), 3.33 (d, J = 6.9 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.89–6.03 (m, 1H), 6.68–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 29.2, 29.4, 29.4, 29.6, 29.6, 29.7, 29.7, 31.9, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): *m/z* calcd for $C_{22}H_{37}O_2$ $[M+H]^+$: 333.2794; found: 333.2798.

*4.2.2.13. 4-Allyl-1-hexadecyloxy-2-methoxy-benzene (**15**).* Method B1: Amount of eugenol (0.094 g; 0.573 mmol), NaH (0.027 g; 0.69 mmol), 1-Bromohexadecane (0.21 ml; 0.69 mmol) and DIAD (0.148 g; 0.73 mmol). Solvent system for column

chromatography: 4% ethyl acetate in hexane. Yield: 76.1%; Appearance: White semi-solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.25–1.46 (m, 26H), 1.82 (quintet, J = 7.2 Hz, 2H), 3.33 (d, J = 6.9 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.11 (m, 2H), 5.89–6.03 (m, 1H), 6.69–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 29.2, 29.4, 29.4, 29.6, 29.6, 29.7, 29.7, 31.9, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 132.6, 137.8, 146.9, 149.4. HRMS (ESI+): m/z calcd for $C_{26}H_{45}O_2$ $[M+H]^+$: 389.3420; found: 389.3426.

*4.2.2.14. 4-Allyl-2-methoxy-1-octadec-9-enyloxy-benzene (**16**).* Method B: Amount of eugenol (0.086 g, 0.523 mmol), Oleyl chloride (0.3 g, 1.045 mmol), K_2CO_3 (0.289 g, 2.092 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 60.3%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.44 (m, 23H), 1.80–1.85 (m, 2H), 1.98–2.02 (m, 3H), 3.33 (d, J = 6.6 Hz, 2H), 3.85 (s, 3H), 3.98 (t, J = 6.9 Hz, 2H), 5.04–5.12 (m, 2H), 5.35 (t, J = Hz, 2H), 5.89–6.03 (m, 1H), 6.68–6.82 (m, 3Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 26.0, 27.2, 29.3, 29.3, 29.4, 29.5, 29.5, 29.7, 29.8, 29.8, 31.9, 39.8, 56.0, 69.2, 112.4, 113.1, 115.6, 120.4, 129.8, 130.0, 132.6, 137.7, 146.9, 149.4. HRMS (ESI+): m/z calcd for $C_{28}H_{47}O_2$ $[M+H]^+$: 415.3576: found: 415.3580.

*4.2.2.15. Acetic acid 4-allyl-2-methoxy-phenyl ester (**17**).* Method A2: Amount of Eugenol (0.1 g; 0.6 mmol), Pyridine (0.095 g; 1.2 mmol), acetic anhydride (0.075 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 67.5%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 2.30 (s, 3H), 3.37 (d, J = 6.6 Hz, 2H), 3.82 (s, 3H), 5.07–5.13 (m, 2H), 5.89–6.02 (m, 1H), 6.75–6.79 (m, 2Ar-H), 6.96 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 20.7, 40.1, 55.8, 112.7, 116.2, 120.7, 122.5, 137.1, 138.0, 139.0, 150.9, 169.3. HRMS (ESI+): m/z calcd for $C_{12}H_{15}O_3$ $[M+H]^+$: 207.1021; found: 207.1024.

*4.2.2.16. 4-Fluoro-benzoic acid 4-allyl-2-methoxy-phenyl ester (**18**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), 4-Fluorobenzoic acid (0.169 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 79.2%; m.p: 68–70° C; Appearance: white solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.41 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 5.08–5.11 (m, 1H), 5.12–5.17 (m, 1H), 5.92–6.05 (m, 1H), 6.80 (d, J = 1.8 Hz, 1Ar-H), 6.83 (s, 1Ar-H), 7.06 (d, J = 7.8 Hz, 1Ar-H), 7.13–7.21 (m, 2Ar-H), 8.20–8.26 (m, 2Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.1, 55.9, 112.8, 115.5, 115.8, 116.2, 120.8, 122.6, 132.9, 133.0, 137.1, 138.1, 139.2, 151.0, 164.0, 164.4, 167.8. HRMS (ESI+): m/z calcd for $C_{17}H_{16}FO_3$ $[M+H]^+$: 287.1083; found: 287.1081.

*4.2.2.17. 2-Chloro-benzoic acid 4-allyl-2-methoxy-phenyl ester (**19**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), 2-chlorobenzoic acid (0.094 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 81.0%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.40 (d, J = 6.9 Hz, 2H), 3.83 (s, 3H), 5.07–5.10 (m, 1H), 5.11–5.16 (m, 1H), 5.91–6.05 (m, 1H), 6.80 (d, J = 1.8 Hz, 1Ar-H), 6.84 (s, 1Ar-H), 7.10 (d, J = 8.1 Hz, 1Ar-H), 7.35–7.40 (m, 1Ar-H), 7.44–7.53 (m, 2Ar-H), 8.09 (dd, J = 1.5, 6.0 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.1, 55.9, 112.9, 116.2, 120.8, 122.6, 126.7, 129.3, 131.2, 132.1, 133.0, 134.4, 137.1, 138.0, 139.3, 151.0, 163.6. HRMS (ESI+): m/z calcd for $C_{17}H_{16}$ ClO_3 $[M+H]^+$: 303.0788; found: 303.0789.

*4.2.2.18. 4-Nitro-benzoic acid 4-allyl-2-methoxy-phenyl ester (**20**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), 4-nitrobenzoic acid (0.102 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 62.4%; Appearance: White solid; m.p: 82–84 °C. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.42 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 5.10 (t, J = 1.2 Hz, 1H), 5.14–5.17 (m, 1H), 5.92–6.06 (m, 1H), 6.83 (d, J = 2.1 Hz, 1Ar-H), 6.85 (s, 1Ar-H), 7.07 (d, J = 7.8 Hz, 1Ar-H), 8.33–8.41 (m, 4Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.1, 55.9, 112.9, 116.3, 120.8, 122.3, 123.6, 131.4, 135.0, 136.9, 137.7, 139.7, 150.7, 150.8, 163.0. HRMS (ESI+): m/z calcd for $C_{17}H_{16}NO_5$ $[M+H]^+$: 314.1028; found: 314.1030.

*4.2.2.19. 3-Nitro-benzoic acid 4-allyl-2-methoxy-phenyl ester (**21**).* Method C2: Amount of Eugenol (0.094 g, 0.58 mmol), DMAP (0.007 g, 0.058 mmol), EDCI.HCl (0.144 g, 0.754 mmol), *m*-Nitrobenzoic acid (0.107 g, 0.638 mmol) and THF (3 ml). Yield: 31.8%; Appearance: White semi-solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.42 (d, J = 6.9 Hz, 2H), 3.81 (s, 3H), 5.11–5.17 (m, 2H), 5.93–6.06 (m, 1H), 6.83–6.85 (m, 2H), 7.08 (d, J = 7.8 Hz, 1H), 7.72 (t, J = 8.1 Hz, 1H), 8.47–8.55 (m, 2H), 9.05 (t, J = 1.8 Hz, 1H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.1, 55.8, 112.9, 116.3, 120.8, 122.4, 125.3, 127.8, 129.8, 131.1, 135.9, 137.0, 137.7, 139.6, 148.4, 150.8, 162.8. HRMS (ESI+): m/z calcd for $C_{17}H_{16}NO_5$ $[M+H]^+$: 314.1028; found: 314.1025.

*4.2.2.20. 1H-indole-3-carboxylic acid 4-allyl-2-methoxy-phenyl ester (**22**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), indole-3-carboxylic acid (0.097 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 22% ethyl acetate and hexanes. Yield: 74.6%; m.p: 168–170 °C; Appearance: white solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.41 (d, J = 6.9 Hz, 2H), 3.81 (s, 3H), 5.09–5.10 (m, 1H), 5.11–5.16 (m, 1H), 5.92–6.06 (m, 1H), 6.81 (d, J = 1.8 Hz, 1Ar-H), 6.84 (s, 1Ar-H), 7.11 (d, J = 8.1 Hz, 1Ar-H), 7.26–7.31 (m, 2Ar-H), 7.43–7.48 (m, 1Ar-H), 8.10 (d, J = 3.0 Hz, 1Ar-H), 8.26 (q, J = 3.0 Hz, 1Ar-H), 8.70 (bs, 1N-H). ^{13}C NMR (75 MHz) (Acetone-d_6) – δ (ppm) 41.8, 57.3, 114.2, 114.9, 117.2, 122.3, 122.4, 123.0, 123.4, 123.6, 124.8, 125.3, 128.5, 134.8, 138.9, 139.7, 140.6, 153.9, 164.3. HRMS (ESI+): m/z calcd for $C_{19}H_{18}NO_3$ $[M+H]^+$: 308.1287; found: 308.1291.

*4.2.2.21. Phenyl-acetic acid 4-allyl-2-methoxy-phenyl ester (**23**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), phenylacetic acid (0.082 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 89.2%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.36 (d, J = 6.6 Hz, 2H), 3.74 (s, 3H), 3.88 (s, 2H), 5.06 (s, 1H), 5.10 (d, J = 7.2 Hz, 1H), 5.90–6.01 (m, 1H), 6.72 (s, 1Ar-H), 6.76 (s, 1Ar-H), 6.91 (d, J = 7.8 Hz, 1Ar-H), 7.33 (m, 5Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.1, 41.0, 55.8, 112.8, 116.2, 120.7, 122.4, 127.1, 128.6, 128.8, 129.4, 129.5, 133.8, 137.1, 138.1, 139.0, 150.9, 169.8. HRMS (ESI+): m/z calcd for $C_{18}H_{19}O_3$ $[M+H]^+$: 283.1334; found: 283.1332.

*4.2.2.22. Phenoxy-acetic acid 4-allyl-2-methoxy-phenyl ester (**24**).* Method B2: Amount of Eugenol (0.1 g; 0.6 mmol), DMAP) (0.007 g; 0.06 mmol), phenoxyacetic acid (0.091 g; 0.6 mmol), DCC (0.151 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate and hexanes. Yield: 93.2%; m.p: 55–57 °C; Appearance: white waxy solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 3.38 (d, J = 6.6 Hz, 2H), 3.80 (s, 3H), 4.90 (s, 2H), 5.07 (t, J = 1.5 Hz, 1H), 5.10–5.14 (m, 1H), 5.88–6.00 (m, 1H), 6.76 (d, J = 1.8 Hz, 1Ar-H), 6.79 (s, 1Ar-H), 6.96–7.04 (m, 4Ar-H), 7.30–7.36 (m, 2Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 40.0, 55.8, 65.2, 112.7, 114.8, 116.2, 120.7, 121.8, 122.2, 129.5, 136.9, 137.3, 139.4, 150.6, 157.8, 167.2.

HRMS (ESI+): *m/z* calcd for $C_{18}H_{19}O_4$ $[M+H]^+$: 299.1283; found: 299.1287.

*4.2.2.23. Propionic acid 4-allyl-2-methoxy-phenyl ester (**25**).* Method A: Amount of eugenol (0.1 g; 0.6 mmol), pyridine (0.095 g; 1.2 mmol) and propionic anhydride (0.095 g; 0.73 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 88.1%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.27 (t, J = 7.5 Hz, 3H), 2.60 (q, J = 8.4 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 5.06–5.09 (m, 1H), 5.10–5.14 (m, 1H), 5.89–6.03 (m, 1H), 6.75 (d, J = 2.1 Hz, 1Ar-H), 6.78 (t, J = 1.8 Hz, 1Ar-H), 6.94 (d, J = 8.4 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 9.2, 27.4, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.9, 150.9, 172.7. HRMS (ESI+): *m/z* calcd for $C_{13}H_{17}O_3$ $[M+H]^+$: 221.1178: found: 221.1184.

*4.2.2.24. Butyric acid 4-allyl-2-methoxy-phenyl ester (**26**).* Method A: Amount of eugenol (0.094 g; 0.58 mmol), pyridine (0.137 g; 1.74 mmol) and butyric anhydride (0.138 g; 0.87 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 72.3%; Appearance: colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.05 (t, J = 7.5 Hz, 3H), 1.73–1.86 (m, 2H), 2.55 (t, J = 7.2 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 13.6, 18.6, 35.9, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.9, 150.9, 171.9. HRMS (ESI+): *m/z* calcd for $C_{14}H_{19}O_3$ $[M+H]^+$: 235.1334: found: 235.1336.

*4.2.2.25. Pentanoic acid 4-allyl-2-methoxy-phenyl ester (**27**).* Method C: Amount of eugenol (0.094 g, 0.58 mmol), pentanoic acid (0.071 g, 0.695 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.144 g, 0.754 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 85.8%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.97 (t, J = 7.2 Hz, 3H), 1.42–1.49 (m, 2H), 1.70–1.80 (m, 2H), 2.57 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.9 Hz, 2H), 3.80 (s, 3H), 5.07–5.13 (m, 2H), 5.91–6.00 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.91–6.94 (m, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 13.8, 22.2, 27.1, 33.8, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.9, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{15}H_{21}O_3$ $[M+H]^+$: 249.1491: found: 249.1490.

*4.2.2.26. Hexanoic acid 4-allyl-2-methoxy-phenyl ester (**28**).* Method C: Amount of eugenol (0.108 g, 0.697 mmol), hexanoic acid (0.1 g, 0.861 mmol), DMAP (0.005 g, 0.043 mmol) and EDCI.HCL (0.197 g, 1.03 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 77.4%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.93 (t, J = 7.2 Hz, 3H), 1.36–1.44 (m, 4H), 1.74–1.79 (m, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.10 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 8.7 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.0, 22.4, 24.8, 31.2, 34.0, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.8/, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{16}H_{23}O_3$ $[M+H]^+$: 263.1647: found: 263.1649.

*4.2.2.27. Heptanoic acid 4-allyl-2-methoxy-phenyl ester (**29**).* Method C2: Amount of eugenol (0.124 g, 0.768 mmol), heptanoic acid (0.1 g, 0.768 mmol), DMAP (0.009 g, 0.076 mmol) and EDCI.HCl (0.176 g, 0.922 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 43.4%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.91 (t, J = 6.9 Hz, 3H), 1.26–1.47 (m, 6H), 1.76 (quintet, J = 7.5 Hz, 2H), 2.57 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.80 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.5, 25.0, 28.8, 31.5, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.9, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{17}H_{25}O_3$ $[M+H]^+$: 277.1804: found: 277.1801.

*4.2.2.28. Octanoic acid 4-allyl-2-methoxy-phenyl ester (**30**).* Method C: Amount of eugenol (0.095 g, 0.58 mmol), octanoic acid (0.1 g, 0.695 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.144 g, 0.754 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 85.2%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.87–0.92 (m, 3H), 1.26–1.47 (m, 8H), 1.76 (quintet, J = 7.2 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.9 Hz, 2H), 3.80 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.6, 25.1, 29.0, 29.0, 31.7, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{18}H_{27}O_3$ $[M+H]^+$: 291.1960: found: 291.1963.

*4.2.2.29. Nonanoic acid 4-allyl-2-methoxy-phenyl ester (**31**).* Method C: Amount of eugenol (0.09 g, 0.552 mmol), nonanoic acid (0.097 g, 0.613 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.152 g, 0.797 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 55.2%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.86–0.93 (m, 3H), 1.29–1.44 (m, 10H), 1.76 (quintet, J = 7.2 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.9, 2H), 3.80 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 25.1, 29.1, 29.2, 29.3, 31.8, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.6, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{19}H_{29}O_3$ $[M+H]^+$: 305.2117: found: 305.2110.

*4.2.2.30. Decanoic acid 4-allyl-2-methoxy-phenyl ester (**32**).* Method C: Amount of eugenol (0.09 g, 0.552 mmol), nonanoic acid (0.097 g, 0.613 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.152 g, 0.797 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 54.5%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.86–0.91 (m, 3H), 1.22–1.42 (m, 12H), 1.76 (quintet, J = 7.5 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.60 (d, J = 6.6 Hz, 2H), 3.08 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 25.1, 29.1, 29.3, 29.5, 31.9, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{20}H_{31}O_3$ $[M+H]^+$: 319.2273: found: 319.2270.

*4.2.2.31. Dodecanoic acid 4-allyl-2-methoxy-phenyl ester (**33**).* Method C: Amount of eugenol (0.094 g, 0.58 mmol), dodecanoic acid acid (0.14 g, 0.638 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.152 g, 0.797 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 58.3%; Appearance: Colourless liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.41 (m, 16H), 1.75 (quintet, J = 7.5 Hz, 2H), 2.56 (t, J = 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.80 (s, 3H), 5.07–5.14 (m, 2H), 5.89–5.14 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 25.1, 29.1, 29.3, 29.4, 29.5, 29.6, 31.9, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): *m/z* calcd for $C_{22}H_{35}O_3$ $[M+H]^+$: 347.2586: found: 347.2592.

*4.2.2.32. Hexadecanoic acid 4-allyl-2-methoxy-phenyl ester (**34**).* Method C2: Amount of eugenol (0.094 g, 0.58 mmol), palmitic acid (0.149 g, 0.58 mmol), DMAP (0.007 g, 0.058 mmol) and EDCI.HCL (0.144 g, 0.754 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 74.6%; Appearance: Colourless

liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 6.9 Hz,3H), 1.23–1.44 (m, 24H), 1.76 (quintet, J = 7.2 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.80 (s, 3H), 5.07–5.14 (m, 2H), 5.89–6.03 (m, 1H), 6.74–6.78 (m, 2Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 25.1, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.7, 31.9, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): m/z calcd for $C_{26}H_{43}O_3$ $[M+H]^+$: 403.3212: found: 403.3209.

*4.2.2.33. Octadec-8-enoic acid 4-allyl-2-methoxy-phenyl ester (**35**).* Method B2: Eugenol (0.1 g; 0.6 mmol), DMAP (0.007 g; 0.06 mmol), DCC (0.151 g; 0.73 mmol) and Oleic acid (0.169 g; 0.6 mmol). Solvent system for column chromatography: 4% ethyl acetate in hexane. Yield: 91.4%; Appearance: pale yellow liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 0.88 (t, J = 3.0 Hz, 3H), 1.26 (bs, 12H), 1.33 (bs, 8H), 1.71–1.78 (m, 2H), 2.02 (bs, 3H), 2.56 (t, J = 7.5 Hz, 2H), 3.37 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 5.08–5.09 (m, 1H), 5.10–5.14 (m, 1H), 5.89–6.03 (m, 1H), 6.75 (d, J = 1.8 Hz, 1Ar-H), 6.78 (s, 1Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 14.1, 22.7, 25.1, 27.2, 27.2, 29.1, 29.2, 29.3, 29.3, 29.5, 29.6, 29.7, 29.7, 29.8, 31.5, 31.9, 34.1, 40.1, 55.8, 112.7, 116.1, 120.7, 122.5, 129.8, 130.0, 137.1, 138.1, 138.8, 150.9, 172.1. HRMS (ESI+): m/z calcd for $C_{28}H_{44}O_3$ $[M+H]^+$: 428.3290; found: 428.3286.

*4.2.2.34. L-pyrrolidine-2-carboxylic acid 4-allyl-2-methoxy-phenyl ester (**36**).* Method B2: Amount of Eugenol (0.2 g; 1.2 mmol), DMAP (0.014 g; 0.12 mmol), (*tert*-butoxycarbonyl)proline (0.258 g; 1.2 mmol), DCC (0.302 g; 1.46 mmol), HCl gas. Solvent system for column chromatography: 25% ethyl acetate and hexanes with 0.1% triethylamine. Yield: 74.3%; Appearance: yellow liquid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.85–2.36 (m, 6H), 3.31 (d, J = 6.6 Hz, 2H), 3.52–3.57 (m, 1H), 3.88 (s, 3H), 5.04–5.11 (m, 2H), 5.91–6.00 (m, 1H) 6.67–6.70 (m, 2Ar-H) 6.85 (d, J = 4.2 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 23.3, 27.7, 39.9, 45.2, 55.9, 60.6, 111.2, 114.3, 115.5, 121.2, 131.9, 137.8, 143.9, 146.5, 166.4. HRMS (ESI+): m/z calcd for $C_{15}H_{20}NO_3$ $[M+H]^+$: 262.1443: found: 262.1448.

*4.2.2.35. Piperidine-4-carboxylic acid 4-allyl-2-methoxy-phenyl ester (**37**).* Method B2: Amount of Eugenol (0.2 g; 1.2 mmol), DMAP (0.014 g; 0.12 mmol), (*tert*-butoxycarbonyl)piperidine-4-carboxylic acid (0.275 g; 1.2 mmol), DCC (0.302 g; 1.46 mmol), HCl gas. Solvent system for column chromatography: 25% ethyl acetate and hexanes with 0.1% triethylamine. Yield: 68.1%; m.p: 144–146 °C; Appearance: pale yellow solid. ^{1}H NMR (300 MHz) ($CDCl_3$) – δ (ppm) 1.72–1.86 (m, 2H), 2.37 (q, J = 5.4 Hz, 2H), 2.98–3.01 (m, 1H), 3.11–3.26 (m, 2H), 3.40 (d, J = 6.6 Hz, 2H), 3.42–3.44 (m, 2H), 3.84 (s, 3H), 5.09 (s, 1H), 5.13 (d, J = 6.3 Hz, 1H), 5.90–6.03 (m, 1H), 6.77 (s, 1Ar-H), 6.80 (s, 1Ar-H), 6.93 (d, J = 7.8 Hz, 1Ar-H). ^{13}C NMR (75 MHz) ($CDCl_3$) – δ (ppm) 8.7, 24.4, 37.3, 40.1, 42.1, 45.9, 55.8, 112.5, 116.2, 120.7, 122.1, 136.9, 137.5, 139.3, 150.4, 171.1. HRMS (ESI+): m/z calcd for $C_{16}H_{22}NO_3$ $[M+H]^+$: 276.1600; found: 276.1598.

*4.2.2.36. L-2-Amino-3-(1H-indol-3-yl)-propionic acid 4-allyl-2-methoxy-phenyl ester (**38**).* Method B2: Amount of Eugenol (0.2 g; 1.2 mmol), DMAP (0.014 g; 0.12 mmol), (*tert*-butoxycarbonyl) tryptophan (0.365 g; 1.2 mmol), DCC (0.302 g; 1.46 mmol), HCl gas. Compound was obtained as HCl salt. Yield: 81.7%; m.p: 132–134 °C; Appearance: off white solid. ^{1}H NMR (300 MHz) (DMSO-d_6) – δ (ppm) 3.37 (d, J = 6.9 Hz, 2H), 3.65 (s, 1H), 3.84 (s, 3H), 4.48 (s, 1H), 5.06 (d, J = 6.3 Hz, 1H), 5.14 (d, J = 1.5 Hz, 1H), 5.90–6.04 (m, 1H), 6.76 (dd, J = 1.8, 8.4 Hz, 1Ar-H), 6.87 (d, J = 8.1 Hz, 1Ar-H), 6.99–7.14 (m, 4Ar-H), 7.35–7.41 (m, 2Ar-H), 7.69 (d, J = 7.8 Hz, 1N-H), 8.82 (bs,3N-H), 11.16 (bs,1N-H). ^{13}C NMR (75 MHz) (DMSO-d_6) – δ (ppm) 26.3, 52.6, 52.7, 55.7, 106.5, 111.5, 113.0, 116.1, 118.6, 120.2, 121.1, 122.2, 125.0, 127.0, 136.2, 136.6, 137.3, 139.5, 150.2, 167.6, 169.7. HRMS (ESI+): m/z calcd for $C_{21}H_{24}Cl_2N_2O_3$ $[M+H]^+$: 422.1164; found: 422.1157.

4.3. Activity of eugenol derivatives

4.3.1. Animals, parasites and ethics statement

BALB/c mice were obtained from central animal facility, SASTRA University, Thanjavur. *Leishmania donovani* (MHOM/IN/AG/83) was maintained *in vitro* in RPMI1640 medium (Gibco), supplemented with 10% FBS (Gibco), and the virulence was maintained by passage through BALB/c mice with *i.v.* infection of stationary phase promastigotes (2×10^7/mice). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All the experimental animal protocols received prior approval from the Institutional Animal Ethical Committee (SASTRA University, Registration Number: 817/PO/ReRc/S/04/CPCSEA; Dated: 20.11.2015).

4.3.2. Promastigote killing assay

Antipromastigote activity was determined by MTT assay. Promastigotes were seeded into 96-well flat bottom plate (Genetix) at 1×10^6 parasites per well in a final volume of 200 μL medium and incubated with an increasing concentrations of synthetic eugenol derivatives (0–250 μg/ml) or known antileishmanials. Parasites were incubated at 22 °C for 48 h followed by MTT assay was done. Absorbance values were determined at 570 nm using a microplate reader (Biotek, Synergy H1). Promastigotes viability was determined as a relative percentage to untreated control sets [25].

4.3.3. Amastigote killing assay

Mouse (BALB/c) peritoneal macrophages were cultured in 8 well chamber glass slide (Genetix), at a cell density of 1×10^5 cells per 200 μl of RPMI1640 supplemented with 10% FBS. Peritoneal macrophages were allowed to attach overnight and were infected with promastigotes at a macrophage: promastigote ratio of 1:10 for 6 h at 37 °C with 5% CO_2. Non-internalized promastigotes were removed by washing twice with RPMI1640 media. The infected macrophages were treated with increasing concentrations of eugenol derivatives (0–250 μg/ml) for another 42 h. Untreated and treated infected macrophages were washed, fixed in methanol and stained with Giemsa stain. Parasite load was calculated as no of amastigotes/100 macrophages under light microscope (Nikon Eclipse Ci) [15,26].

4.3.4. Cytotoxicity assay

RAW264.7 murine macrophage cells and BALB/c derived peritoneal macrophages (1×10^5) were cultured in 96 well plate with increasing concentrations of eugenol derivatives (0–250 μg/ml) in 200 μl of RPMI media supplemented with 10% FBS at 37 °C in 5% CO_2 atmosphere. After 48 h of incubation the culture media was replaced with fresh RPMI (without phenol red and FBS) containing 0.5 mg/ml MTT and incubated for 3 h at 37 °C. Then HCl-isopropanolic solution (0.04 M HCl in Isopropanol) was added to each well. After 15 min of incubation at room temperature, absorbance of solubilized MTT formazan product was colorimetrically measured at 570 nm using a microplate reader (Biotek, Synergy H1) [26,27].

4.3.5. In vivo anti-visceral leishmanial response

Male BALB/c mice (6–8 weeks, weight matched) were remained uninfected or infected with 2×10^7 *L. donovani* promastigotes intravenously. Uninfected control (n = 4) and infected control mice (n = 4) were injected intravenously via the tail-vein with

phosphate-buffered saline. Another two infected groups (n = 4 per group) were treated with comp. **35** (10 mg/kg of body weight (B. W.) and 25 mg/kg of B. W.). All the treatments were started two weeks post infection with five days interval between each treatment. After completion of four weeks treatment, all the mice were sacrificed and hepatic and splenic parasite burden were determined from tissue imprints after Giemsa staining. Results were expressed as Leishman Donovan Units (LDU) as described earlier [15,26].

4.3.6. Nitrite generation assay

Nitrite generation was estimated by using Griess reagent (sigma). The cell free supernatants were collected from different *in vitro* and *in vivo* experimental sets after indicated time points. Then 100 μl of cell supernatant was mixed with 100 μl of Griess reagent and incubated in dark for 30 min. Then absorbance was read at 550 nm using a microplate reader (Biotek, Synergy H1). The nitrite accumulation was calculated from the nitrite standard curve prepared from $NaNO_2$ as the substrate and the data were expressed in micromoles of nitrite [15,26].

4.3.7. Arginase 1 activity

Arginase 1 activity was measured in peritoneal macrophages and splenocyte lysates as described by Adhikari et al. [15] Uninfected macrophages, infected macrophages and comp.**35** [1 μg/ml (2.33 μM), 2.5 μg/mL (5.82 μM), and 5 μg/mL (11.65 μM)] treated infected macrophages were lysed after 48 h with 0.1% Triton X-100 with 25 mM Tris–HCl. Arginase was activated by heating at 56 °C and arginine hydrolysis was carried out using 0.5 M L-arginine (pH 9.7) at 37 °C for 15–20 min. The urea concentration was measured at 540 nm after the addition of α-isonitrosopropiophenone followed by heating at 95 °C for 30 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 mol of urea/min. Similarly, splenocyte lysates from *in vivo* sets were used to determine the arginase 1 activity [15].

4.3.8. IL-10 and IL-12 level by ELISA

Cell free supernatants of peritoneal macrophages (*in vitro*) and splenocytes (*in vivo*) were collected after 24 h from uninfected, *L. donovani* infected and comp.**35** [2.5 μg/ml (5.82 μM)] treated *L. donovani* infected sets. Cytokines in the indicated culture supernatants were assayed using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions [26].

4.3.9. Isolation of RNA, mRNA expression study

Total RNA extracted from uninfected, infected and comp. **35** [2.5 μg/ml (5.82 μM)] treated infected peritoneal macrophages after 6 h (TRIZOL; Invitrogen). Similarly, total RNA was extracted from the splenocytes cultures of individual *in vivo* experimental groups re-stimulated with soluble leishmanial antigen (5 μg/ml) for 6 h 1 μg of total RNA from different experimental sets were reverse transcribed using Revert Aid M-MuLV Reverse Transcriptase (Fermentas) to synthesis the cDNA. cDNA was amplified with green *Taq*DNA polymerase (Fermentas) in 25 μL reaction mixture using specific primers of *iNOS2*, *arginase 1*, *IL-12p40*, *IL-10* and *IFN-γ* using Veriti Thermal Cycler (Applied Biosystems). Sequences of the PCR primers are listed in Table 5. The reaction conditions consisted of an initial activation step (5 min at 95 °C) and a cycling step (denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and extension for 1 min at 72 °C for 35 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was also done to ensure equal cDNA input. PCR-amplified product was subsequently run on 1.2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light and analyzed in BioRad gel documentation system. In real time PCR, (Eppendorf RealPlex master cycler, using 2X SYBR premix Ex Taq II (TAKARA Bio), the mRNA expression levels of the target genes were normalized against those of GAPDH levels and expressed as relative fold change compared with untreated controls and quantified by the 2^{-ddCT} method [26,28,29].

Table 5
Primer sequences.

Gene	Primer sequences
iNOS2	Forward 5′-CAG AGG ACC CAG AGA CAA GC-3'; Reverse 5′-AAG ACC AGA GGC AGC ACA TC-3';
Arginase 1	Forward 5′-CAG AAG AAT GGA AGA GTC AG-3'; Reverse 5′-CAG ATA TGC AGG GAG TCA CC-3';
IL-12 p40	Forward 5′-CAC GCC TGA AGA AGA TGA CA-3'; Reverse 5′-GAC AGA GAC GCC ATT CCA CA-3';
IL-10	Forward 5′-TCA CTC TTC ACC TGC TCC AC-3'; Reverse 5′- CTA TGC TGC CTG CTC TTA CTC-3; '
IFN-γ	Forward 5' – GGA TAT CTG GAG GAA CTG GC-3′ Reverse 5′- CGA CTC CTT TTC CGC TTC CT -3′
GAPDH	Forward 5′-GAG CCA AAC GGG TCA TCA TC-3'; Reverse, 5′-CCT GCT TCA CCA CCT TCT TG-3';

4.4. Statistical analysis

All the *in vitro* experiments were performed in triplicates and *in vivo* experiments with four male BALB/c mice per group. The data, represented as mean values ± standard error of means (S.E.M). 50% effective concentration (EC_{50}) and 50% inhibitory concentration (IC_{50}) values were calculated from non-linear regression curve using the statistical software Origin 6.1, Northampton, MA, USA. The therapeutic index (TI) was calculated by the ratio of cytotoxicity with EC_{50} [30]. One-way ANOVA was employed to assess the significance of the differences between the mean values of uninfected control, infected and treated-infected groups.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.08.030.

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