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Original article

Novel natural product-based cinnamates and their thio and thiono analogs as potent inhibitors of cell adhesion molecules on human endothelial cells

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ABSTRACT

In the present study, we report the design and synthesis of novel analogs of cinnamates, thiocinnamates and thionocinnamates and evaluated the potencies of these analogs to inhibit TNF-α induced ICAM-1 expression on human endothelial cells. By using whole cell-ELISA, our screening data demonstrated that ethyl 3′,4′,5′-trimethoxythionocinnamate (ETMTC) is the most potent inhibitor of TNF-α induced ICAM-1, VCAM-1 and E-selectin. As functional consequences, ETMTC abrogated TNF-α induced adhesion of neutrophils to the endothelial monolayer. Structure–activity relationship studies revealed the critical role of the chain-length of the alkyl group in the alcohol moiety, number of methoxy groups in the aromatic ring of the cinnamoyl moiety and the presence of the α, β- C–C double bond in the thiocinnamates and thionocinnamates.

1. Introduction

Leukocyte mediated inflammatory response is the key event in multiple inflammatory diseases including asthma, acute respiratory distress syndrome, rheumatoid arthritis, and atherosclerosis [1–3]. Leukocytes in the inflamed tissue secrete proinflammatory mediators, reactive oxygen species and proteases that cause tissue damage, inflammation and disease pathogenesis [4–6]. Pharmaceutical companies are designing drugs to limit leukocyte mediated inflammatory response. However, current anti-inflammatory drugs show limited efficacy and exhibit severe side effects. Therefore, more specific and potent anti-inflammatory drugs are needed urgently [7].

The endothelium plays a crucial role in inflammation by providing key signals for leukocyte migration and extravasation. In response to tissue injury caused by environmental toxicants (pathogens and non-pathogens), endothelial cells are activated by extravascular stimuli inducing the surface expression of cell adhesion molecules (CAMs) that include E-selectin, ICAM-1 and VCAM-1 [8]. Interaction of CAMs on the surface of endothelial cell and leukocytes facilitates rolling and extravasations of leuckocytes to the sites of inflammation. Therefore, pharmacological inhibition of CAMs on endothelial cells is a promising strategy for therapeutic intervention of inflammatory disorders [9].

Previously, we have reported the isolation and characterization of a novel aromatic ester from the chloroform extract of *Piper longum* that exhibited potent ICAM-1 inhibitory activity on human umbilical vein endothelial cells [10]. In another study on coumarins/thionocoumarins, we have shown that the sulfur analogs (thionocoumarins), when compared to the oxygenated analogs (coumarins), are more potent inhibitors of TNF-α induced expression of ICAM-1 on endothelial cells [11]. In view of these findings, in the present study, we have designed, synthesized and evaluated the ICAM-1 inhibitory activity of libraries of cinnamates, thiocinnamates

Abbreviations: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor κB; HUVECs, human umbilical cord vein endothelial cells; ETMC, ethyl 3′,4′,5′-trimethoxycinnamate; ETMTC, ethyl 3′,4′,5′-trimethoxythionocinnamate.

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and thionocinnamates. We for the first time report novel thio and thiono analogs of the new cinnamic acid esters that exhibit potent cell adhesion molecules inhibitory activity on human endothelial cells. Further, detailed structure–activity relationship of these novel cinnamates reveal that the chain-length of the alkyl group in the alcohol moiety, substituents in the aromatic ring and the α, β- C–C double bond of the thionocinnamates have significant effect on the inhibition of TNF-α induced expression of ICAM-1 on endothelial cells.

2. Biological results

2.1. Cinnamates are non-toxic to the endothelial cells

All cinnamate analogs were examined for their cytotoxic effect on human endothelial cells as described in the experimental procedure section, and were found to be non-toxic to endothelial cells (data not shown) as more than 95% cells were viable at maximum tolerable concentrations. This implied that these derivatives are safe to use at indicated concentrations. The maximal tolerable concentration was found to be different for different compounds. For all further analyses, the maximum tolerable concentration was used as shown in Tables 1–3.

2.2. Cinnamates, thiocinnamates and thionocinnamates inhibit TNF-α induced expression of ICAM-1 on endothelial cells

Cinnamates, thiocinnamates and thionocinnamates were evaluated for their ability to inhibit TNF-α induced expression of ICAM-1 using high-throughput whole cell-ELISA assay. Endothelial cells were pre-incubated for 2 h with or without cinnamates, thiocinnamates and thionocinnamates at various concentrations followed by induction with TNF-α (10 ng/mL) for 16 h. There was 3-fold higher induction in ICAM-1 expression by TNF-α compared to unstimulated cells (data not shown). However, when endothelial cells were pre-treated with cinnamates, thiocinnamates and thionocinnamates at various sub-lethal concentrations there was a significant inhibition in TNF-α induced expression of ICAM-1. The IC_{50} value of each compound was calculated (Tables 1–3) separately from its respective activity–concentration graph. Also, the levels of maximum % inhibition of ICAM-1 expression are reported at their maximum tolerable concentration where cells viability was not affected by the tested compounds (Tables 1–3). Of the 36 compounds tested, ethyl 3′,4′,5′-trimethoxythionocinnamate (ETMTC, **54**) was found to be the most potent inhibitor of the TNF-α induced ICAM-1 expression. Furthermore, the inhibition of ICAM-1 by ETMTC was concentration dependent (Fig. 1).

Table 1
ICAM-1 expression inhibitory activities of the cinnamates **10**–**17** and **20**–**23**.

Entry	Compound	Conc. (μg/mL)[a]	% Inhibition	IC_{50} Concentration (μg/mL)
1	DMSO (Vehicle)	0.25%	No	No
2	Isopropyl 3′,4′,5′ -trimethoxycinnamate (**10**)	65	80	45
3	Phenyl 3′,4′,5′ -trimethoxycinnamate (**11**)	45	40	–
4	Propyl 2′,5′ -dimethoxycinnamate (**12**)	120	35	–
5	Propyl 2′,5′ -dimethoxycinnamate (**13**)	120	55	110
6	Phenyl 2′,5′ -dimethoxycinnamate (**14**)	60	30	–
7	Isopropyl 2′-hydroxy-3′ -methoxycinnamate (**15**)	120	30	–
8	Octyl cinnamate (**16**)	70	35	–
9	Benzyl cinnamate (**17**)	70	50	70
10	Propyl 3′,4′,5′ -trimethoxydihydrocinnamate (**20**)	120	62	110
11	Isopropyl 3′,4′,5′ -trimethoxydihydrocinnamate (**21**)	65	40	–
12	Propyl 3′,4′ -dimethoxydihydrocinnamate (**22**)	100	55	90
13	Isopropyl 3′,4′ -dimethoxydihydrocinnamate (**23**)	110	30	–

'–' These compounds did not reach up to 50% inhibition even at maximum tolerable concentration.
[a] Highest concentration tested that showed more than 95% cells viability.

Table 2
ICAM-1 expression inhibitory activities of the thiocinnamates **29**–**44**.

Entry	Compound	Conc. (μg/mL)[a]	% Inhibition	IC_{50} (μg/mL)
1	Ethyl 3′,4′,5′-trimethoxythiocinnamate (**29**)	30	65	25
2	Propyl 3′,4′,5′-trimethoxythiocinnamate (**30**)	70	50	70
3	Octyl 3′,4′,5′-trimethoxythiocinnamate (**31**)	70	46	–
4	Phenyl 3′,4′,5′-trimethoxythiocinnamate (**32**)	50	85	30
5	Benzyl 3′,4′,5′-trimethoxythiocinnamate (**33**)	40	75	20
6	Ethyl 2′,5′-dimethoxythiocinnamate (**34**)	50	70	35
7	Propyl 2′,5′-dimethoxythiocinnamate (**35**)	50	55	45
8	Octyl 2′,5′-dimethoxythiocinnamate (**36**)	50	30	–
9	Benzyl 2′,5′-dimethoxythiocinnamate (**37**)	60	50	60
10	Ethyl thiocinnamate (**38**)	50	55	47
11	Octyl thiocinnamate (**39**)	60	45	–
12	Phenyl thiocinnamate (**40**)	70	65	55
13	Benzyl thiocinnamate (**41**)	60	40	–
14	Ethyl 3′,4′,5′-trimethoxydihydro -thiocinnamate (**42**)	60	50	60
15	Octyl 3′,4′,5′-trimethoxydihydro -thiocinnamate (**43**)	80	40	–
16	Benzyl 3′,4′,5′-trimethoxydihydro -thiocinnamate (**44**)	60	45	–

'–' These compounds did not reach up to 50% inhibition even at maximum tolerable concentration.
[a] Highest concentration tested that showed more than 95% cells viability.

2.3. ETMTC inhibits TNF-α induced expression of VCAM-1 and E-selectin

Next, we investigated the effect of ETMTC on the TNF-α induced expression of two other important cell adhesion molecules, VCAM-1 and E-selectin by using whole cell-ELISA assay. Human endothelial cells were pre-treatment for 2 h followed by induction with TNF-α for 4 h to measure E-selectin and 16 h to measure

Table 3
ICAM-1 expression inhibitory activities of the thionocinnamates **54**–**61**.

Entry	Compound	Conc. (μg/mL)[a]	% Inhibition	IC_{50} (μg/mL)
1	Ethyl 3′,4′,5′ -trimethoxythionocinnamate (**54**)[b]	20	95	10
2	Propyl 3′,4′,5′ -trimethoxythionocinnamate (**55**)	30	80	17
3	Ethyl 2′,5′ -dimethoxythionocinnamate (**56**)	25	80	15
4	Propyl 2′,5′ -dimethoxythionocinnamate (**57**)	40	70	25
5	Ethyl thionocinnamate (**58**)	50	90	22
6	Propyl thionocinnamate (**59**)	80	75	50
7	Isopropyl thionocinnamate (**60**)	35	80	20
8	Butyl thionocinnamate (**61**)	85	70	55

[a] Highest concentration tested that showed more than 95% cells viability.
[b] Thionocinnamate ETMTC **54** was prepared by using the natural ETMC **47** from *Piper longum*.

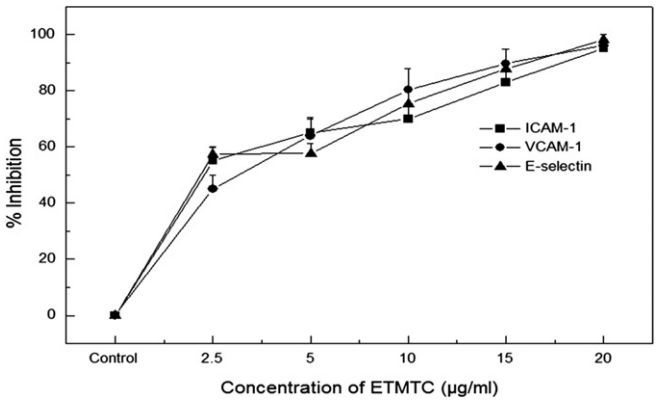


Fig. 1. Concentration dependent inhibition of TNF-α induced ICAM-1, VCAM-1 and E-selectin expression on endothelial cells by ETMTC. Endothelial cells were incubated with or without indicated concentration of ETMTC for 2 h followed by co-incubation with or without TNF-α (10 ng/mL). E-selectin was measured after 4 h while ICAM-1 and VCAM-1 were measured after 16 h by cell based-ELISA as described in experimental procedure. Data are mean ± SD of 3 independent experiments. Significant compared to vehicle control at $P < 0.05$.

VCAM-1. Pretreatment of endothelial cells with ETMTC significantly attenuated TNF-α induced expression of VCAM-1 on endothelial cells. The inhibition of VCAM-1 by ETMTC was concentration dependent (Fig. 1). Likewise, ETMTC pre-treatment markedly inhibited the TNF-α induced expression of E-selectin on endothelial cells (Fig. 1). Similar inhibition pattern of adhesion molecules on endothelial cells was observed by ETMTC in response to LPS (data not shown).

The inhibitory activity of ETMTC on ICAM-1, VCAM-1 and E-selectin expression was further confirmed by flow cytometry. In response to TNF-α stimulation, a substantial increase (5–6 folds) in the expression of all these three adhesion molecules was observed (Fig. 2A–C). However, pre-treatment of endothelial cells with ETMTC markedly inhibited the TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin (by more than 95%, Fig. 2A–C).

2.4. ETMTC inhibits neutrophils adhesion to endothelial monolayer

Next we assessed the functional implications of inhibition of adhesion molecules by ETMTC using neutrophil-endothelial adhesion assay. Endothelial cells were incubated with or without ETMTC at various concentrations for 2 h prior to induction with TNF-α (10 ng/mL) for 6 h. As measured by colorimetric assay, unstimulated endothelial cells showed low adherence of neutrophils, however there was a three-fold increase in neutrophil adhesion in response to TNF-α stimulation (data not shown). The pre-treatment of endothelial cells by ETMTC significantly inhibited the TNF-α induced neutrophil adhesion in concentration dependent manner. The maximum inhibition of TNF-α induced neutrophils adhesion was calculated by more than 95% (Fig. 3).

3. Discussion

We have identified ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**, ETMTC) as most potent inhibitor of TNF-α induced cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) from a series of thirty six cinnamate esters studied in the present report. *Ex-vivo* inhibition of neutrophils adhesion to endothelial monolayer by ETMTC substantiated its anti-inflammatory function. Clinical studies using monoclonal antibodies directed against either VCAM-

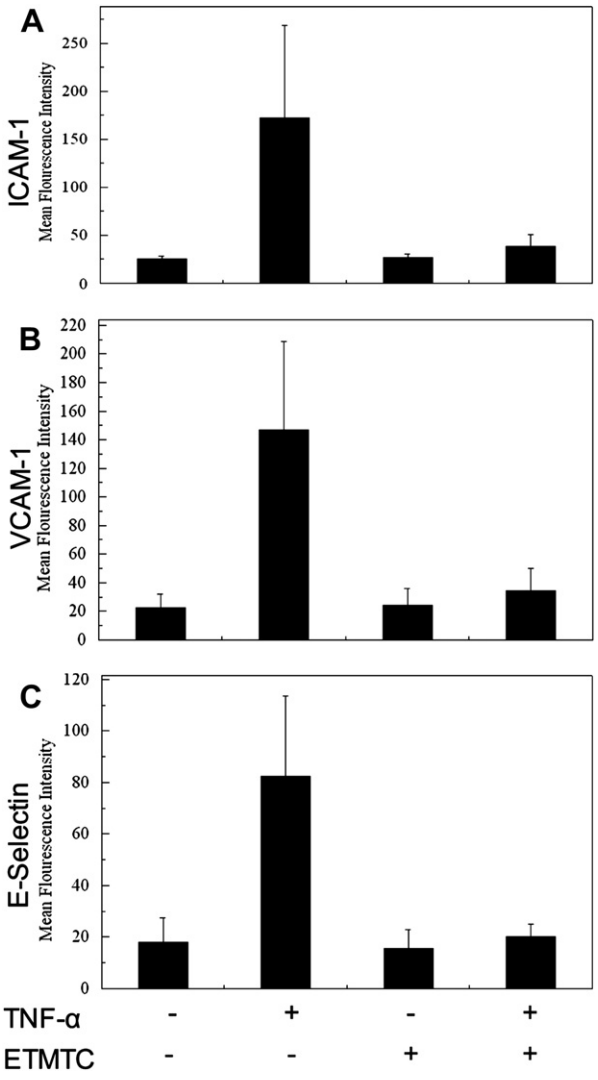


Fig. 2. Flow cytometric analysis of inhibition of TNF-α induced ICAM-1, VCAM-1 and E-selectin expression on endothelial cells by ETMTC: Endothelial cells were pretreated with or without ETMTC (20 μg/mL) for 2 h followed by TNF-α (10 ng/mL) stimulation for 4 h for E-selectin measurement and 16 h for ICAM-1 and VCAM-1 measurement by flow cytometry as described in experimental procedures. The data presented as mean + S.D. of three independent experiments. Cell Quest software was used for statistical analysis ($P < 0.05$).

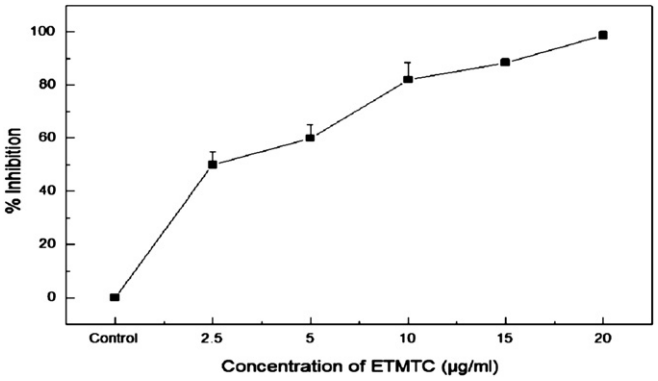


Fig. 3. ETMTC inhibits adhesion of neutrophils to endothelial monolayer. Endothelial cells were grown to confluency and incubated with or without ETMTC at various concentrations. After 2 h, cells were stimulated with TNF-α (10 ng/mL) for 6 h. Adherent neutrophils were assayed colorimetrically as described in experimental procedure. Values shown are mean ± SD of 3 independent experiments.

1 and/or ICAM-1 have indicated critical role of these two adhesion molecules in inhibiting neutrophil adhesion and subsequent protection from inflammatory response in multiple inflammatory diseases including organ transplantation. On the contrary, our lead small molecule, ETMTC coordinatively inhibits ICAM-1, VCAM-1 and E-selectin expression that may result in robust protection from inflammation.

To examine the role of different modifications, *i.e* (i) effect of alkyl chain in the alcohol part of esters, (ii) number of methoxy groups in the benzene ring, (iii) saturation of the α, β- alkene bond in the cinnamoyl moiety and (iv) replacement of hetero atom (sulfur in place of oxygen in the ester moiety; both in the carbonyl part in the cinnamoyl moiety to give the thiono esters and the oxygen of the alkoxy group in the alcoholic moiety to give the thio esters) in inhibiting the TNF-α induced expression of ICAM-1, we have synthesized different oxygenated cinnamates **10**–**17** and **20**–**23** (Fig. 4), and the corresponding thiocinnamates **29**–**44** (Fig. 5) and the thionocinnamates **54**–**61** (Fig. 6). The experimental activity data shown in Tables 1–3 revealed that the following functional components are critical for the ICAM-1 expression inhibitory activities among all the three tested classes of compounds.

3.1. Oxygenated cinnamates *10*–*17* and *20*–*23*

3.1.1. Effect of the length of the alkyl group in the alcohol moiety

The ICAM-1 inhibitory activity data given in Table 1 revealed that activity of the cinnamates **10**–**17** and **20**–**23** significantly decreases with the increase in the length of the alkyl chain in the alcohol part. Earlier we have reported that ethyl cinnamate (**51**), propyl cinnamate (**52**) and the butyl cinnamate (**53**), having increasingly longer alkyl chains in the alcohol moiety inhibit 85%, 70% and 55%, respectively (Table 4, Fig. 7) the TNF-α induced expression of ICAM-1 in human endothelial cells [10]. In the present study, we have found that further increase in the chain length, *i.e.* the octyl cinnamate (**16**) having eight carbon atoms in the alkyl chain showed insignificant ICAM-1 expression inhibitory activity (35% inhibition only, Table 1). Similarly, the ICAM-1 expression inhibitory activity of ethyl 3′,4′,5′-trimethoxycinnamate (**47**) having two carbon atom alkyl group in the alcohol moiety is much higher than of propyl 3′,4′,5′-trimethoxycinnamate (**48**) having three carbon atom alkyl group (90% and 62% ICAM-1 expression inhibition values, respectively, Table 4, Fig. 7)

Further, the esters having branched alkyl chains or those carrying aryl moieties had low-to-moderate ICAM-1 expression inhibitory activity values as revealed by the % inhibition values of ICAM-1 expression for these esters in Table 1.

3.1.2. Effect of the number of methoxy groups in the benzene ring

The ICAM-1 inhibitory activity decreases with the decrease in the number of methoxy groups present in the benzene ring of cinnamates **10**–**17** and **20**–**23**. Thus, the ICAM-1 expression inhibition value of isopropyl 3′,4′,5′-trimethoxycinnamate (**10**, 80%) having three methoxy groups is higher than that of the propyl 2′,5′-dimethoxycinnamate (**13**, 55% inhibition) having two methoxy groups, further the compound propyl 2′-hydroxy-3′-methoxycinnamate (**15**) having only one methoxy group shows only 30% ICAM-1 expression inhibition (Table 1). Also the esters lacking any methoxy group in the benzene ring have very low activity as ICAM-1 expression inhibitors (Table 1).

3.1.3. Effect of the saturation of the alkene bond

It has been observed that the dihydro derivatives of cinnamates were less effective in inhibiting the TNF-α induced expression of ICAM-1. Thus, as can be seen in Table 1, the ICAM-1 expression inhibition value of isopropyl 3′,4′,5′-trimethoxycinnamate (**10**, 80%) is higher than that of its dihydro analog, *i.e.* the isopropyl 3′,4′,5′-trimethoxydihydrocinnamate (**21**, 40%). Similarly, the ICAM-1 expression inhibitory activity of ethyl 3′,4′,5′-trimethoxycinnamate (**47**, 90%) is much higher than that of its dihydro analog, ethyl 3′,4′,5′-trimethoxydihydrocinnamate (**62**, 50%, Table 4, Fig. 7). Again the ICAM-1 expression was inhibited to the extent of 55% by propyl 2′,5′-dimethoxycinnamate (**13**) and its dihydro analog, isopropyl 3′,4′-dimethoxydihydrocinnamate (**23**) has the inhibitory activity value of 30% only (Table 1)

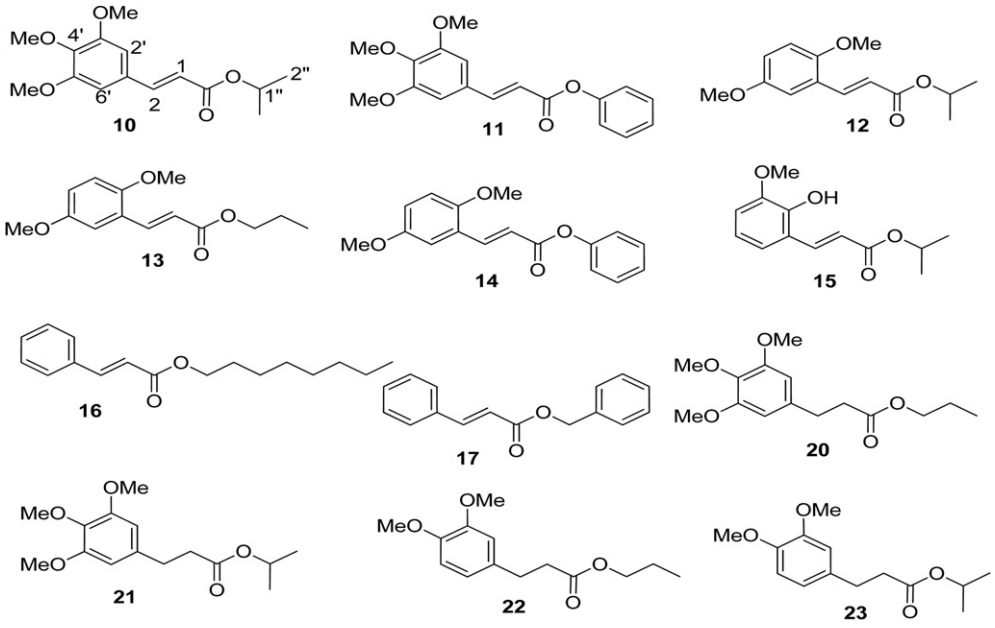


Fig. 4. Structures of alkyl and aryl cinnamates.

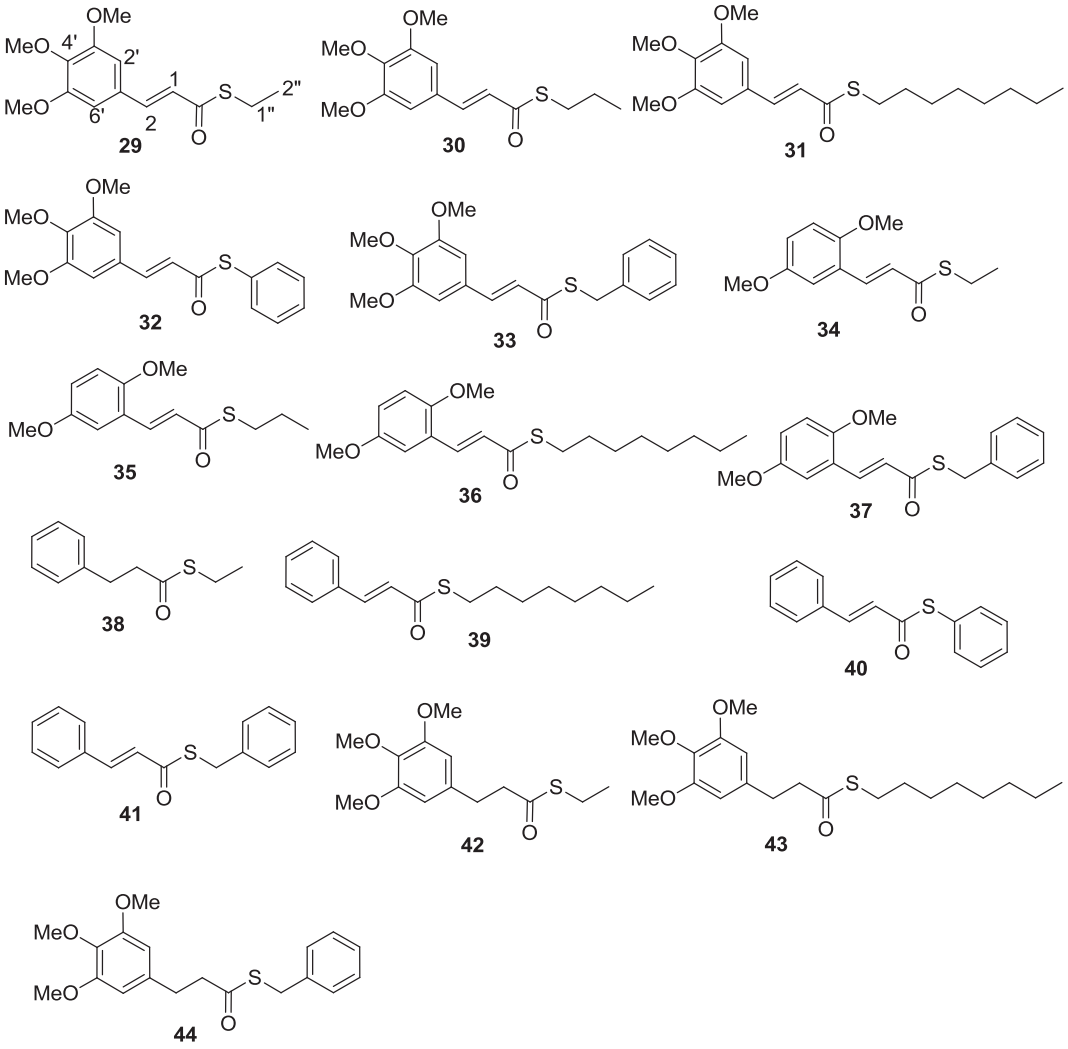


Fig. 5. Structures of alkyl and aryl thiocinnamates.

3.2. Thiocinnamates ***29–44***

3.2.1. Effect of the length of the alkyl groups in the alcohol moiety

It is very clearly evident from Table 2 that ICAM-1 inhibitory activity decreases with an increase in the length of the alkyl chain in the alcohol moiety in the thiocinnamates **29–44**. A significant fall in the ICAM-1 inhibitory activities was observed in increasing the chain length from ethyl to propyl to octyl in the alcohol part of the thiocinnamate esters **29–31**, **s**imilar trend was observed in the compounds **34–36**, **38–39** and **42–43** also (Table 2).

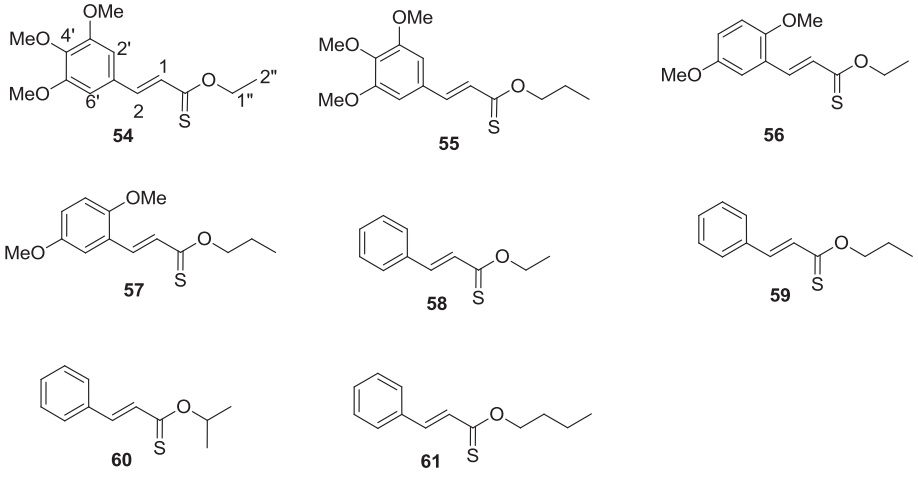


Fig. 6. Structures of alkyl and aryl thionocinnamates.

Table 4
ICAM-1 expression inhibitory activities of the compounds already reported by us [10] for comparison to establish the SAR study in the current manuscript.

Compound	Concentration (μg/mL)[a]	% ICAM-1 expression inhibition	IC_{50} (μg/mL)
Ethyl 3′,4′,5′-tri -methoxycinnamate (ETMC, **47**)	50	90	25
Propyl 3′,4′,5′ -trimethoxycinnamate (**48**)	120	62	100
Ethyl cinnamate (**51**)	130	85	95
Propyl cinnamate (**52**)	140	70	100
Butyl cinnamate (**53**)	140	55	125
Ethyl 3′,4′,5′-trimethoxy -dihydrocinnamate (**62**)	110	50	110

[a] Highest concentration tested that showed more than 95% cells viability.

3.2.2. Effect of the number of methoxy groups in the benzene ring

As revealed from Table 2, the ICAM-1 inhibitory activity decreases with the decrease in the number of methoxy groups present in the benzene ring of the thiocinnamates **29**–**44**. The IC_{50} value of ethyl 3′,4′,5′-trimethoxythiocinnamate (**29**, 25 μg/mL) is lower than that of the ethyl 2′,5′-dimethoxythiocinnamate (**34**, 35 μg/mL). The lack of the presence of any methoxy groups in the benzene ring further decreases the ICAM-1 expression lowering activity as the IC_{50} value (50 μg/mL) of ethyl thiocinnamate (**38**) lacking any methoxy group is much higher as compared to those of compounds **29** (25 μg/mL) and **34** (35 μg/mL) having three and two methoxy groups, respectively (Table 2).

3.2.3. Effect of the saturation of the alkene bond

The dihydro analogs **42**, **43** and **44** of the thiocinnamates **29**, **31** and **33**, respectively when tested for ICAM-1 inhibitory activity were found to be much less effective in inhibiting the TNF-α induced expression of ICAM-1 (Table 2).

*3.3. Thionocinnamates **54**–**61***

3.3.1. Effect of the length of the alkyl group in the alcohol moiety

Again the same trend as in the cases of cinnamates **10**–**17** and **20**–**23** and the thiocinnamates **29**–**44** was observed in the case of the thionocinnamates **54**–**61** as well, viz. the ICAM-1 inhibitory activity of the compounds **54**–**61** decreases with the increase in the length of the alkyl group of the alcohol moiety. Thus the IC_{50} value of ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**, ETMTC, 10 μg/mL), ethyl 2′,5′-dimethoxythionocinnamate (**56**, 15 μg/mL) and ethyl thionocinnamate (**58**, 22 μg/mL) are lower than those of their corresponding propyl and butyl analogs **55** (IC_{50} 17 μg/mL), **57** (IC_{50} 25 μg/mL), and **59** (IC_{50} 50 μg/mL) and **61** (IC_{50} 55 μg/mL), respectively (Table 3).

3.3.2. Effect of the number of methoxy groups in the benzene ring

As revealed from Table 3, similar to the cases of the cinnamates **10**–**17** and **20**–**23** and thiocinnamates **29**–**44**, the ICAM-1 inhibitory activity values of the thionocinnamates **54**–**61** decrease with the decrease in the number of methoxy groups present in the benzene rings of the thionocinnamates as well. Thus, the IC_{50} values of the ethyl thionocinnamates: ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**, ETMTC, 10 μg/mL) having three methoxy groups is lower than that of the ethyl 2′,5′-dimethoxythionocinnamate (**56**, 15 μg/mL) having two methoxy groups, and that of ethyl thionocinnamate (**58**, 22 μg/mL) lacking the presence of any methoxy group (Table 3). The same trend prevailed in the case of propyl thionocinnamates, thus the IC_{50} value of propyl 3′,4′,5′-trimethoxythionocinnamate having three methoxy groups (**55**, 17 μg/mL) is lower than that of propyl 2′,5′-dimethoxythionocinnamate having two methoxy groups (**57**, 25 μg/mL), which in turn is lower than that of propyl thionocinnamate (**59**, 50 μg/mL) lacking the presence of any methoxy group (Table 3).

We have earlier reported that the replacement of the lactone ring carbonyl oxygen atom by sulfur in the case of coumarins makes the corresponding thionocoumarins more potent than the parent coumarins in inhibiting the ICAM-1 expression [11]. Taking clue from this finding, we have in the present study, synthesized new analogs **10**–**17** and **20**–**23** and different novel thio and thiono analogs **29**–**44** and **54**–**61**, respectively of the naturally occurring cinnamate **47** [10]. On comparing the ICAM-1 inhibitory activity values of the new oxygen containing cinnamate analogs **10**–**17** and **20**–**23** of the natural cinnamate **47** (Table 1) with those of the thiocinnamates (Table 2) and thionocinnamates (Table 3), we have observed that sulfur containing compounds are more potent than their oxygen containing counterparts. When the oxygen atom of the alkoxy moiety was replaced by sulfur atom, the resulting thiocinnamates **29**–**44** (Table 2) showed better ICAM-1 expression inhibition than the corresponding oxygenated cinnamates **10**–**17** and **20**–**23** (Table 1). However, the ICAM-1 expression lowering activities of the thionocinnamates **54**–**61** having the ketonic oxygen of the cinnamoyl moiety substituted by the sulfur atom, unlike the substitution of the alkoxy oxygen of the alcoholic chain by sulfur to give the thiocinnamates **29**–**44** (Table 2) were much higher (Table 3). A brief comparison of the ICAM-1 inhibitory activity of cinnamates, thiocinnamates and thionocinnamates is presented in Fig. 8, it can clearly be seen that there is inhibition in ICAM-1 expression, shown by the three different classes of cinnamates follows the order: thionocinnamates > thiocinnamates > the oxygenated cinnamates. Further it is noteworthy to see that all the

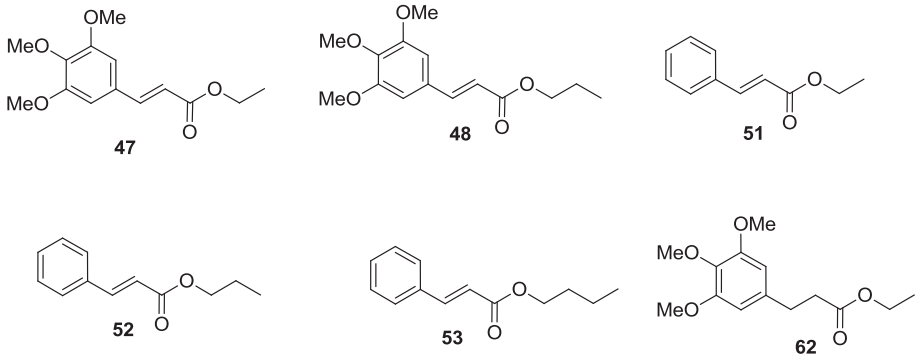


Fig. 7. Structures of the compounds already reported by us earlier [10] for comparison to establish SAR study in the current manuscript.

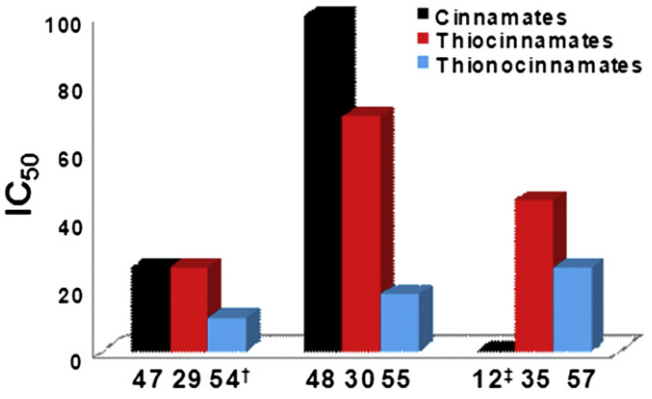


Fig. 8. Comparison of ICAM-1 inhibitory activity of selected cinnamates, thiocinnamates and thionocinnamates. IC_{50} values of some cinnamates, thiocinnamates and thionocinnamates are compared to demonstrate the effect of replacement of oxygen atom with that of sulfur. Compound numbers are shown on *x*-axis and IC_{50} values are represented on *y*-axis. † The most active compound (**54**) was prepared from the natural product **47** isolated from *Piper longum*. ‡ did not show any IC_{50} values

eight thionocinnamates **54**–**61** showed very high activities as they all exhibited % inhibition of ICAM-1 expression values in the range 70–95% with IC_{50} values ranging between 10 and 55 μg/mL (Table 3), as compared to those of the thiocinnamates **29**–**44** (having % inhibition values ranging between 30 and 85% with IC_{50} values ranging between 20 and 70 μg/mL and only ten compounds out of sixteen showed the IC_{50} values, Table 2) and those of the oxygenated cinnamates **10**–**17** and **20**–**23** having % inhibition values ranging between 30 and 80% and the IC_{50} values ranging between 70 and 110 μg/mL and only five compounds out of twelve showed the IC_{50} values (Table 1), In other words, all the thionocinnamates **54**–**61** were moderate to highly active as each of the eight thionocinnamates showed significant values of % inhibition of ICAM-1 expression and IC_{50} values (Table 3) unlike the thiocinnamates **29**–**44** (Table 2) and the oxygenated cinnamates **10**–**17** and **20**–**23** (Table 1). Taken together, our results suggest that the ICAM-1 expression inhibitory activity of the thionocinnamate esters is better when:

(i) thionocinnamate ester has alcohol moiety of shorter chain length (ethyl is the best),
(ii) more number of methoxy groups are present in the aromatic ring,
(iii) the α,β-unsaturation is present in the cinnamoyl moiety, and
(iv) sulfur atom is present in the place of oxygen atom in the ketonic moiety, *i.e.* the cinnamoyl part of the ester (viz. the thionocinnamates).

The efficacy of our thiono derivative ETMTC (**54**) was found to be 200 times more than that of the natural parent compound from which it was prepared, present in *P. longum*, *i.e.* ETMC (**47**) [10]. In short, thionocinnamates and thiocinnamates have better inhibitory activities than cinnamates because of sulfur in their structures. It has been well documented in literature that sulfur-containing compounds are biologically more active then oxygen containing counterparts [12,13]. As members of the same periodic group, sulfur and oxygen share similarities in chemical reactivity. However, sulfur's position lower in the periodic table endows its compounds with distinct properties that are advantageous to biological systems. For example, thiols are superior nucleophiles compared with alcohols and also serve as versatile activating groups in thioester biochemistry. Disulfide bonds (RS–SR) are more stable than peroxide bonds (RO–OR), and biology has taken advantage of this stability by using disulfide bonds as structural features of proteins. The presence of thiols in their structures could be responsible for their better biological activities including antioxidant properties [14,15]. Furthermore, in comparison with the known anti-inflammatory clinical drugs, such as aspirin (IC_{50} 6000 μM), mesalamine (IC_{50} 16,000 μM) and phenyl methimazole (IC_{50} 500 μM), ETMTC (IC_{50}, 40 μM) showed greater potency in inhibiting the TNF-α induced expression of adhesion molecules [16–18]. Likewise, when compared with the clinically used anti-inflammatory drugs, such as diclofenac, *N*-acetylcysteine and pyrrolidone dithiocarbamate that are effective at concentrations of 750 μM, 100 μM and 1000 μM, respectively, [19,20] ETMTC (**54**) shows robust inhibition at very low concentration. Summing up these results indicate that our novel compound (ETMTC, **54**) is potentially effective and therefore is, worthy of further pharmaceutical studies.

Previously, we have reported that inhibition of cell adhesion molecules by different natural compounds was mediated by NF-κB [21–23]. Although, in the current study, the mechanism of cell adhesion molecule expression inhibition by ETMTC and its analogs is not investigated, it is tempting to speculate that they may be interfering with the NF-κB activation pathway. Furthermore, nuclear factor E2p45-related factor (Nrf2) is a central transcription factor that regulates the anti-oxidant defense system and acts as a modifier of several inflammatory diseases that involve oxidative stress and inflammation. Nrf2 activating agents are also known for NF-κB inhibitors [24–26]. These findings prompt us to examine the effect of cinnamate analogs on Nrf2 activation. Our unpublished data from ARE-reporter based assay showed a similar structure–activity pattern for Nrf2 activation by these esters. In any event, the exact mechanism of action responsible for inhibition of cell adhesion molecules expression remains to be elucidated and would be the subject of a future publication.

4. Conclusion

Our results of identifying ethyl 3′,4′,5′-trimethoxythionocinnamate (ETMTC, **54**) and its analogs have implications in discovering therapeutically valuable lead molecules against various inflammatory diseases. Importantly, these findings will open new avenues for research on ethyl 3′,4′,5′-trimethoxythionocinnamate as a lead pharmaceutical molecule.

5. Experimental procedures

5.1. Reagents and chemicals

The organic solvents (dioxane, petroleum ether and ethyl acetate) used were dried and distilled prior to their use. All other chemical like alcohols, thiols and cinnamic/dihydrocinnamic acids used were purchased from Sigma–Aldrich, USA and were used without any further purification. The analytical TLCs were performed on precoated Merck silica gel 60 F_{254} plates; the spots were visualized under UV light. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance at 300 MHz and 75.5 MHz, respectively using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (*J*) are in Hz. The HRMS were recorded on a JEOL JMS-AX505W instrument. Anti-ICAM-1, E-selectin and TNF-α were purchased from Pharmingen, USA. M-199 media, L-glutamine, endothelial cell growth factor, trypsin, Pucks saline, HEPES, *o*-phenylenediamine and anti-mouse IgG-HRP were purchased from Sigma Chemical Co., USA. Fetal calf serum was purchased from Biological Industries, Israel.

5.2. Chemistry

5.2.1. General procedure for the synthesis of the cinnamates **10**–**17** and **20**–**23**

A mixture of the commercially available cinnamic acid (**4**)/substituted cinnamic acids **1**–**3**/dihydrocinnamic acids (**18**/**19**, 5 mmol) and the corresponding commercially available alcohols **5**–**9**, *i.e.* propyl (**7**)/isopropyl (**5**)/octyl (**8**)/phenyl (**6**)/benzyl alcohol (**9**) (25 mL) and concentrated sulfuric acid (0.2 mL) was refluxed for 4–5 h (Schemes 1 and 2). On completion of the reactions as observed from TLC examination, the reaction mixture was neutralized by the addition of 10% aqueous sodium hydrogen carbonate solution (w/v) and then extracted with chloroform (2 × 25 mL), the combined organic layer was separated and dried over anhydrous Na_2SO_4. The crude product obtained after evaporation of organic solvent was loaded on a small silica gel column and eluted with chloroform to obtain the pure esters in 71–99% yields. The structures of all the cinnamates **10**–**17** and **20**–**23** were unambiguously established on the basis of their spectral (IR, ^{1}H NMR, ^{13}C NMR spectra and HRMS) analysis. The structures of the known esters **11**, **16** and **17** were further confirmed by comparison of their melting points with those reported in the literature [27,28] (Table 5).

5.2.2. Isopropyl 3′,4′,5′-trimethoxycinnamate (**10**)

It was obtained as pale yellow oil in 95% yield. IR (thin film): 2981, 2930, 1713, 1638, 1450, 1311, 1176, 1039, 980, 768 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 [6H, d, J = 6.2 Hz, CH(C*H*$_3$)$_2$], 3.88 (9H, s, 3× OCH_3), 5.14 [1H, m, C*H*(CH$_3$)$_2$], 6.35 (1H, d, J = 15.9, C–2H), 6.75 (2H, s, C-2′H and C-6′H), 7.58 and (1H, d, J = 15.9 Hz, C–1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 21.64 [CH(*C*H$_3$)$_2$], 55.84 (2× OCH_3), 60.62 (OCH_3), 67.46 [O*C*H(CH$_3$)$_2$], 104.99 (C-2′ and C-6′), 117.78 (C-2), 129.73 (C-1′) 140.54 (C-4′), 143.94 (C-21), 153.14 (C-3′ and C-5′) and 166.11 (CO); HRMS calcd for $C_{15}H_{20}O_5$ m/z 280.1311, observed m/z 280.1314.

5.2.3. Isopropyl 2′,5′-dimethoxycinnamate (**12**)

It was obtained as light yellow oil in 97% yield. IR (Thin film): 2966, 1709, 1633, 1497, 1318, 1258, 1174, 1046, 988 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 [6H, d, J = 6.2 Hz, CH(C*H*$_3$)$_2$], 3.89 (6H, s, 2× OCH_3), 5.11 [1H, m, C*H*(CH$_3$)$_2$], 6.37 (1H, d, J = 15.9, C–2H), 6.78 (2H, m, C–3′H and C–4′H), 7.0 (1H, s, C–6′H) and 7.58 (1H, d, J = 15.9 Hz, C–1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 21.71 [CH(*C*H$_3$)$_2$], 56.21 (2× OCH_3), 70.43 [OCH(CH$_3$)$_2$], 111.51 (C-6′), 114.49 (C-4′), 115.20 (C-3′), 116.0 (C-1′), 116.39 (C-2), 138.94 (C-1), 151.3 (C-2′) 152.81 (C-5′) and 166.51 (CO); HRMS calcd for $C_{14}H_{18}O_4$ m/z 250.1205, observed m/z 250.1207.

5.2.4. Propyl 2′,5′-dimethoxycinnamate (**13**)

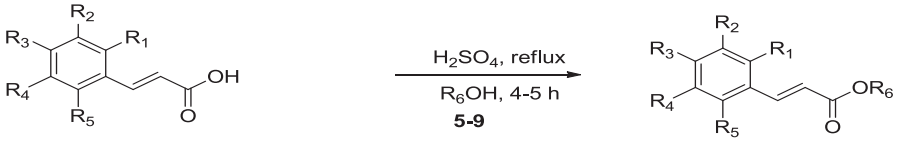
It was obtained as light yellow oil in 96% yield. IR (Thin film): 2982, 1712, 1638, 1496, 1312, 1271, 1176, 1039, 980 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, J = 7.3 Hz, $CH_2CH_2CH_3$), 1.61 (2H, m, $CH_2CH_2CH_3$), 3.83 and 3.85 (6H, s, 2× OCH_3), 4.03 (2H, t, J = 6.7 Hz, $OCH_2CH_2CH_3$), 6.35 (1H, d, J = 15.9, C–2H), 6.77 (3H, m, C–3′H, C-4′ and C-6′) and 7.59 (1H, d, J = 15.9 Hz, C–1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 10.91 ($CH_2CH_2CH_3$), 21.84 ($CH_2CH_2CH_3$), 55.63 and 55.73 (C-2′ and C-5′) 65.84 ($OCH_2CH_2CH_3$), 111.28 (C-6′), 114.62 (C-4′), 115.19 (C-3′), 116.0 (C-1′), 116.22 (C-2), 138.61 (C-1), 151.51 (C-2′), 152.58 (C-5′) and 170.81 (CO); HRMS calcd for $C_{14}H_{18}O_4$ m/z 250.1205, observed m/z 250.1202.

5.2.5. Phenyl 2′,5′-dimethoxycinnamate (**14**)

It was obtained as white solid (mp 92–95 °C) in 99% yield. IR (Thin film): 2837, 1720, 1630, 1494, 1261, 1142, 1046, 988 cm^{-1} ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.81 and 3.71 (6H, s, 2× OCH_3), 6.69 (1H, d, J = 16.08, C–2H), 7.42–6.82 (8H, m, Ar–H), 8.14 (1H, d, J = 16.08 Hz, C–1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.71 and 55.98 (2× OCH_3), 112.45 (C-6′), 113.48 (C-4′), 117.58 (C-3′), 117.94 (C-2), 121.65 (C-2″ and C-6″), 123.59 (C-1′), 125.6 (C-4″), 129.32 (C-3″ and C-5″), 141.71 (C-1), 150.91 (C-1″), 153.49 (C-2′ and C-5′), 165.60 (CO). HRMS calcd for $C_{17}H_{16}O_4$ m/z 284.1049, observed m/z 284.1051.

5.2.6. Isopropyl 2′-hydroxy-3′-methoxycinnamate (**15**)

It was obtained as yellow viscous oil in 8 yield. IR (thin film):2838, 1728, 1590, 1459, 1240, 1109, 1010 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.31 [6H, d, J = 6.2 Hz, CH(C*H*$_3$)$_2$], 3.90 (3H, s, OCH_3), 5.13 [1H, m, OC*H*(CH$_3$)$_2$], 6.27 (1H, d, J = 15.9 Hz, C–2H), 6.80 (1H, d, J = 8.2 Hz, H-4′), 7.02 (1H, dd, J = 8.3 and 1.8 Hz, H-5′), 7.13 (1H, d, J = 1.8 Hz, H-6′) and 7.57 (1H, d, J = 15.9 Hz, C–1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 22.35 CH(*C*H$_3$)$_2$, 56.30 (OCH_3), 68.07 O*C*H(CH$_3$)$_2$, 111.96, 113.47 and 117.12 (C-4′, C-5′ and C-6′), 122.10 (C-2), 128.47 (C-1′), 144.63 (C-1), 146.26 and 148.93 (C-2′ and C-3′) and 167.31 (CO); HRMS calcd for $C_{13}H_{16}O_4$ m/z 236.1049, observed m/z 236.1049.

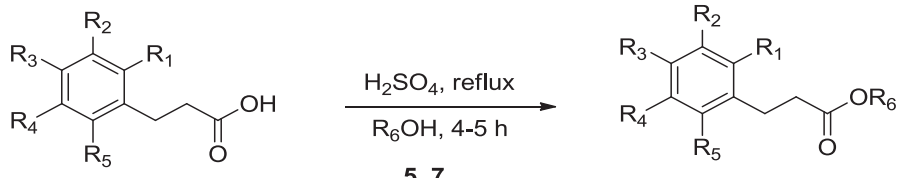


	R_1	R_2	R_3	R_4	R_5
1	H	OCH_3	OCH_3	OCH_3	H
2	OCH_3	H	H	OCH_3	H
3	OH	OCH_3	H	H	H
4	H	H	H	H	H

5 R_6 = $CH(CH_3)_2$
6 R_6 = C_6H_5
7 R_6 = C_3H_7
8 R_6 = C_8H_{17}
9 R_6 = $CH_2C_6H_5$

Product	R_1	R_2	R_3	R_4	R_5	R_6	% Yield
10	H	OCH_3	OCH_3	OCH_3	H	$CH(CH_3)_2$	95
11	H	OCH_3	OCH_3	OCH_3	H	C_6H_5	71
12	OCH_3	H	H	OCH_3	H	$CH(CH_3)_2$	97
13	OCH_3	H	H	OCH_3	H	C_3H_7	96
14	OCH_3	H	H	OCH_3	H	C_6H_5	99
15	OH	OCH_3	H	H	H	$CH(CH_3)_2$	86
16	H	H	H	H	H	C_8H_{17}	86
17	H	H	H	H	H	$CH_2C_6H_5$	95

Scheme 1.



18-19

95-98 %

20-23

	R_1	R_2	R_3	R_4	R_5
18	H	OCH_3	OCH_3	OCH_3	H
19	H	OCH_3	OCH_3	H	H

Product	R_1	R_2	R_3	R_4	R_5	R_6	% Yield
20	H	OCH_3	OCH_3	OCH_3	H	C_3H_7	95
21	H	OCH_3	OCH_3	OCH_3	H	$CH(CH_3)_2$	97
22	H	OCH_3	OCH_3	H	H	C_3H_7	96
23	H	OCH_3	OCH_3	H	H	$CH(CH_3)_2$	98

Scheme 2.

*5.2.7. Propyl 3′,4′,5′-trimethoxydihydrocinnamate (**20**)*

It was obtained as colorless oil in 95% yield. IR (thin film): 1714, 1638, 1451, 1311, 1215, 1067, 980, 756 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, J = 7.3 Hz, $CH_2CH_2CH_3$), 1.64 (2H, m, $CH_2CH_2CH_3$), 2.63 (2H, t, J = 7.9 Hz, C–2H), 2.90 (2H, t, J = 7.5 Hz, C–1H), 3.81 and 3.83 (9H, s, 3× OCH_3), 4.04 (2H, t, J = 6.7 Hz, $OCH_2CH_2CH_3$) and 6.74 (2H, s, C-2′ and C-6′) ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.65 ($CH_2CH_2CH_3$), 22.30 ($CH_2CH_2CH_3$), 31.70 (C-2), 36.34 (C-1), 56.35 (C-3′OCH_3 and C-5′OCH_3), 61.05 (C-4′OCH_3), 66.38 ($OCH_2CH_2CH_3$), 105.63 (C-2′ and C-6′), 136.69 (C-1′), 153.51 (C-3′, C-4′, C-5′) and 173.20 (CO); HRMS calcd for $C_{15}H_{22}O_5$ m/z 282.1467, observed m/z 282.1465.

*5.2.8. Isopropyl 3′,4′,5′-trimethoxydihydrocinnamate (**21**)*

It was obtained as colorless oil in 97% yield. IR (thin film): 1713, 1638, 1451, 1311, 1256, 1172, 980, 768 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.86 [6H, d, J = 6.2 Hz, $CH(CH_3)_2$], 2.23 (2H, t, J = 7.3 Hz, C–2H), 2.53 (2H, t, J = 7.6 Hz, C–1H), 3.45 and 3.48 (9H, s, 3× OCH_3), 4.65 [1H, m, $OCH(CH_3)_2$] and 6.08 (2H, s, C-2′H, and C-6′H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 22.07 [$CH(CH_3)_2$], 31.66 (C-2), 36.57 (C-1), 56.27 (2× OCH_3), 60.95 (OCH_3), 67.91 [$OCH(CH_3)_2$], 105.65 (C-2′ and C-6′), 136.70 (C-1′), 153.45 (C-3′, C-4′, C-5′) and 172.53 (CO). HRMS calcd for $C_{15}H_{22}O_5$ m/z 282.1467, observed m/z 282.1465.

*5.2.9. Propyl 3′,4′-dimethoxydihydrocinnamate (**22**)*

It was obtained as colorless oil in 96% yield. IR (thin film): 1715, 1639, 1455, 1345, 1180, 1062, 979 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, t, J = 7.3 Hz, $CH_2CH_2CH_3$), 1.61 (2H, m, $CH_2CH_2CH_3$), 2.60 (2H, t, J = 7.9 Hz, C–2H), 2.89 (2H, t, J = 7.5 Hz, C–1H), 3.83 and 3.85 (6H, s, 2× OCH_3), 4.03 (2H, t, J = 6.7 Hz, $OCH_2CH_2CH_3$) and 6.77 (3H, m, C-2′, C-5′ and C-6′H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 10.91 ($CH_2CH_2CH_3$), 21.84 ($CH_2CH_2CH_3$), 30.48 (C-2), 36.01 (C-1), 55.63 (C-3′OCH_3), 55.73 (C-4′OCH_3) 65.84 ($OCH_2CH_2CH_3$), 111.28 (C-2′), 111.62 (C-5′), 111.99 (C-6′), 133.11 (C-1′), 147.38 (C-4′), 148.78 (C-3′), 172.81 (CO). HRMS calcd for $C_{14}H_{20}O_4$ m/z 252.1362, observed m/z 252.1361.

*5.2.10. Isopropyl 3′,4′-dimethoxydihydrocinnamate (**23**)*

It was obtained as yellow viscous oil in 98% yield. IR (thin film): 1713, 1638, 1451, 1313, 1173, 1062, 981 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.21 [6H, d, J = 6.2 Hz, $CH(CH_3)_2$], 2.56 (2H, t, J = 8.0 Hz, C–2H), 2.89 (2H, t, J = 7.5 Hz, C–1H), 3.85 and 3.86 (6H, s, 2× OCH_3), 5.02 [1H, m, $OCH(CH_3)_2$] and 6.78 (3H, m, C-2′, C-5′ and C-6′H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 21.60 [$CH(CH_3)_2$], 30.16 (C-2), 36.56 (C-1), 55.55 (C-3′OCH_3), 55.66 (C-4′OCH_3) 67.47 [$OCH(CH_3)_2$], 111.15 (C-2′), 111.53 (C-5′), 111.99 (C-6′), 133.07 (C-1′), 147.27 (C-4′), 148.67 (C-3′) and 172.31 (CO). HRMS calcd for $C_{14}H_{20}O_4$ m/z 252.1362, observed m/z 252.1360.

Table 5
Melting points of the known compounds.

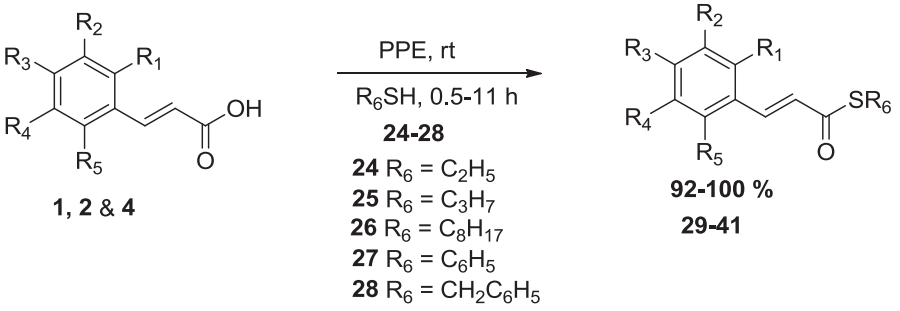
Compound	% Yield	Mp (°C)	
		Lit. (ref.)	Obs.
Phenyl 3′,4′,5′-trimethoxycinnamate (**11**)	71	95–97 °C (*23*)	94–96 °C
Octyl cinnamate (**16**)	86	Oil (*24*)	Oil
Benzyl cinnamate (**17**)	95	33–35 °C (*24*)	Oil
Ethyl thiocinnamate (**38**)	100	Oil (*25*)	Oil
Octyl thiocinnamate (**39**)	94	Oil (*26*)	Oil
Phenyl thiocinnamate (**40**)	98	78–80 °C (*27*)	80–81 °C
Benzyl thiocinnamate (**41**)	92	67–69 °C (*28*)	67–68 °C

*5.3. Synthesis of the thiocinnamates **29**–**44***

A mixture of the commercially available carboxylic acids (**1**, **2**, **4** and **18**, 3 mmol), the appropriate commercially available thiol (**24**–**28**, 3.1 mmol) and polyphosphate ester (PPE, 2 mL) was stirred at room temperature for 0.5–11 h (Schemes 3 and 4). After completion of the reaction, the mixture was treated with saturated aqueous sodium hydrogen carbonate solution (20 mL) and extracted with chloroform (3 × 20 mL). The combined chloroform extract was dried over sodium sulfate and evaporated in a rotary vacuum evaporator. The crude compounds were passed through a silica-gel column, using pure petroleum ether as eluent, the desired compounds were obtained as colorless viscous oils in 92–98% yields. The structures of all the thio esters were unambiguously established on the basis of their spectral (IR, ^1H NMR, ^{13}C NMR spectra and HRMS) analysis. The structure of the known esters **38**–**41** were further confirmed by comparison of their melting points with those reported in the literature [29–33] (Table 5).

*5.3.1. Ethyl 3′,4′,5′-trimethoxythiocinnamate (**29**)*

It was obtained as colorless oil in 98% yield. IR (thin film): 1635 (C=O), 1464, 1421, 1287, 1126, 1005 cm^{-1}; ^1H NMR data (300 MHz, $CDCl_3$); δ 1.31 (3H, t, J = 7.2 Hz, CH_2CH_3), 3.04 (2H, t, J = 7.2 Hz, SCH_2), 3.86 (9H, s, 3× OCH_3), 6.61 (1H, d, J = 15.7 Hz, C–2H), 6.81



Product	R_1	R_2	R_3	R_4	R_5	R_6	% Yield
29	H	OCH_3	OCH_3	OCH_3	H	C_2H_5	98
30	H	OCH_3	OCH_3	OCH_3	H	C_3H_7	98
31	H	OCH_3	OCH_3	OCH_3	H	C_8H_{17}	93
32	H	OCH_3	OCH_3	OCH_3	H	C_6H_5	97
33	H	OCH_3	OCH_3	OCH_3	H	$CH_2C_6H_5$	98
34	OCH_3	H	H	OCH_3	H	C_2H_5	98
35	OCH_3	H	H	OCH_3	H	C_3H_7	97
36	OCH_3	H	H	OCH_3	H	C_8H_{17}	97
37	OCH_3	H	H	OCH_3	H	$CH_2C_6H_5$	98
38	H	H	H	H	H	C_2H_5	100
39	H	H	H	H	H	C_8H_{17}	94
40	H	H	H	H	H	C_6H_5	98
41	H	H	H	H	H	$CH_2C_6H_5$	92

Scheme 3.

(2H, brs, C-2′H and C-6′H) and 7.53 (1H, d, $J = 15.7$ Hz, C—1H). ^{13}C NMR data (75.5 MHz, $CDCl_3$): δ 14.94 (SCH_2CH_3), 22.70 (SCH_2CH_3), 61.11 and 61.26 (3× OCH_3), 105.80 (C-2′ and C-6′), 126.58, 131.76, 138.83 (C-2, C-1′ or/C-4′), 142.42 (C-1), 153.76 (C-3′ and C-5′), 189.94 (C=O); HRMS: Calcd for $C_{14}H_{18}O_4S$ $[M]^+$ 282.1082, found 282.1080.

*5.3.2. Propyl 3′,4′,5′-trimethoxythiocinnamate (**30**)*

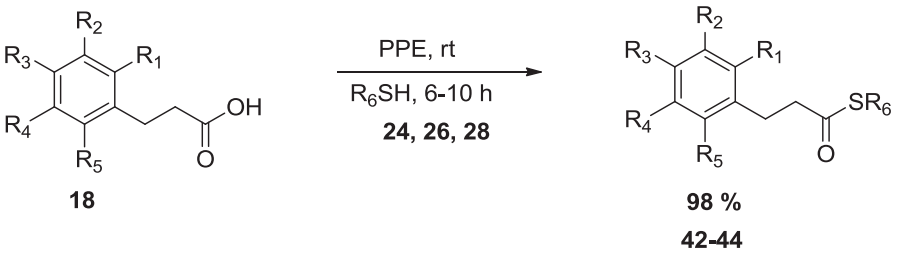
It was obtained as viscous oil in 98% yield. IR (Thin film): 1635 (C=O), 1464, 1421, 1287, 1126, 1005 cm^{-1}; ^{1}H NMR data (300 MHz, $CDCl_3$); δ 1.01 (3H, t, $J = 7.3$ Hz, $CH_2CH_2CH_3$), 1.70 (2H, m, $CH_2CH_2CH_3$), 3.01 (2H, t, $J = 7.8$ Hz, SCH_2), 3.85 (9H, s, 3× OCH_3), 6.62 (1H, d, $J = 15.7$ Hz, C—2H), 6.76 (2H, brs, C-2′H and C-6′H) and 7.52 (1H, d, $J = 15.7$ Hz, C—1H). ^{13}C NMR data (75.5 MHz, $CDCl_3$): δ 13.24 ($CH_2CH_2CH_3$), 22.66 and 30.84 (CH_2CH_3 and SCH_2), 56.07 (3× OCH_3), 105.48 (C-2′ and C-6′), 124.35, 129.51, 138.83 (C-2, C-1′ or/C-4′), 140.11 (C-1), 153.35 (C-3′ and C-5′), 189.54 (C=O); HRMS: Calcd for $C_{15}H_{20}O_4S$ $[M + Na]^+$ 319.0975, found 319.0972.

*5.3.3. Octyl 3′,4′,5′-trimethoxythiocinnamate (**31**)*

It was obtained as colorless viscous oil in 93% yield. IR (Thin film):1664, 1580, 1457, 1341, 1266, 1127, 1034 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, $J = 7.0$ Hz, CH_2CH_3), 1.27—1.55 (2H, m, CH_2CH_3), 2.87—2.89 (10H, m, C-2″H-C-6″H), 3.00 (2H, t, $J = 7.1$ Hz, SCH_2), 3.88 (9H, s, 3× OCH_3), 6.64 (1H, d, $J = 15.8$ Hz, C—2H), 6.76 (2H, s, C-2′H and C-6′H), 7.49 (1H, d, $J = 15.8$ Hz, C—1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.9 (CH_3), 13.92 (CH_2CH_3), 22.87, 23.80, 28.88, 30.42 and 33.4 (C-6″, C-5″, C-4″, C-3″ and C-2″), 38.82 (C-1″), 66.86 (3× OCH_2), 118.29 (C-1), 127.92 (C-2′ and C-6′), 128.73 (C-3′ and C-5′), 130.04 (C-4′), 134.44 (C-2), 144.35 (C-1), 191.98 (CO), HRMS calcd for $C_{20}H_{30}O_4S$ m/z 366.1865, observed m/z 366.1863.

*5.3.4. Phenyl 3′,4′,5′-trimethoxythiocinnamate (**32**)*

It was obtained as green solid mp 74—76 °C in 97% yield. IR (Thin film): 1669, 1581, 1464, 1342, 1266, 1129, 1023 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86—3.89 (9H, s, 3× OCH_3), 6.56 (1H, d, $J = 15.85$ Hz, C—2H), 6.80 (2H, s, C—2′H and C-6′H), 7.17—7.41 (5H, m, C-2″H-C-6″H), 7.75 (1H, d, $J = 15.85$, C—1H); ^{13}C NMR (75.5 MHz,



Product	R_1	R_2	R_3	R_4	R_5	R_6	% Yield
42	H	OCH_3	OCH_3	OCH_3	H	C_2H_5	98
43	H	OCH_3	OCH_3	OCH_3	H	C_8H_{17}	98
44	H	OCH_3	OCH_3	OCH_3	H	$CH_2C_6H_5$	98

Scheme 4.

$CDCl_3$): δ 58.3 and 63.08 (3× OCH_3), 107.76 (C-2′ and C-6′), 118.65 (C-2), 123.77 (C-5″ and C-6″), 127.89 (C-1′), 131.79 (C-2″, C-3″ and C-4″), 143.55 (C-4′), 148.66 (C-1), 153.24 (C-3′ and C-5′), 155.66 (C-1″), 189.45 (CO); HRMS calcd for $C_{18}H_{18}O_4S$ *m/z* 330.0926, observed *m/z* 330.0929.

*5.3.5. Benzyl 3′,4′,5′-trimethoxythiocinnamate (**33**)*

It was obtained as light green oil in 98% yield. IR (Thin film): 1662, 1580, 1454, 1341, 1268, 1126, 1035 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.87–3.88 (9H, m, 3× OCH_3), 4.26 (2H, s, SCH_2), 6.60 (1H, d, *J* = 15.68 Hz, C–2H), 6.75 (2H, s, C-2′H and C-6′H), 7.27–7.33 (5H, m, C-2″H-C-6″H), 7.59 (1H, d, *J* = 15.68, C–1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 35.36 (SCH_2), 58.33 and 63.08 (3× OCH_3), 107.85 (C-2′ and C-6′), 126.03 (C-4″), 129.43 (C-5″ and C-6″), 130.78 (C-2, C-4′, C-2″ and C-3″), 131.65 (C-1′), 139.78 (C-1″), 143.14 (C-3′ and C-5′), 155.64 (C-1), 190.94 (CO); HRMS calcd for $C_{19}H_{20}O_4S$ *m/z* 344.1139, observed 344.1139.

*5.3.6. Ethyl 2′,5′-dimethoxythiocinnamate (**34**)*

It was obtained as light green viscous oil in 98% yield. IR (Thin film): 1663, 1605, 1496, 1464, 1288, 1222, 1140, 1027 cm^{-1}; ^1H NMR data (300 MHz, $CDCl_3$); ^1H NMR data (300 MHz, $CDCl_3$); δ 1.0 (3H, t, *J* = 6.8 Hz, CH_2CH_3), 3.0 (2H, q, *J* = 7.04 Hz, CH_2CH_3), 3.7 (6H, s, 2× OCH_3), 6.7 (1H, s, Ar–H) 6.8 (1H, d, *J* = 12.4 Hz, C–2H), 6.87 (1H, s, Ar–H), 7.0 (1H, s, Ar–H) and 7.8 (1H, d, *J* = 15.9 Hz, C–1H); ^{13}C NMR data (75.5 MHz, $CDCl_3$): δ 14.8 (CH_2CH_3), 23.5 (CH_2CH_3), 56.32(2× OCH_3), 112.7 (C-2), 113 (C-3′), 117.6 (C-6′), 123.9 (C-1′), 126.2 (C-4′), 135.87 (C-1), 153.49 (C-5′), 153.8(C-2′) and 190.4 (C=O); HRMS calcd for $C_{13}H_{16}O_3S$ *m/z* 252.0820, observed 252.0818.

*5.3.7. Propyl 2′,5′-dimethoxythiocinnamate (**35**)*

It was obtained as light green viscous oil in 97% yield. IR (Thin film): 1664, 1607, 1497, 1463, 1289, 1223, 1140, 1030 cm^{-1}; ^1H NMR data (300 MHz, $CDCl_3$); δ 1.0 (3H, t, *J* = 7.2 Hz, $CH_2CH_2CH_3$), 1.66 (2H, m, $CH_2CH_2CH_3$), 2.97 (2H, t, *J* = 7.0 Hz, $CH_2CH_2CH_3$), 3.7 (3H, s, OCH_3), 3.8 (3H, s, OCH_3), 6.77 (1H, d, *J* = 16.0 Hz, C–2H), 6.82–7.02 (3H, m, Ar–H) and 7.89 (1H, d, *J* = 15.9 Hz, C–1H); ^{13}C NMR data (75.5 MHz, $CDCl_3$): δ 13.6 ($CH_2CH_3CH_3$), 13.6($CH_2CH_2CH_3$), 23.3 ($CH_2CH_2CH_3$), 56.0 and 56.3 (2× OCH_3), 112.7 (C-2), 113.6 (C-3′), 117.6 (C-6′), 123.9 (C-1′) 126.2 (C-4′), 135.8 (C-1), 153.5 (C-5′), 153.8 (C-2′) and 190.44 (C=O); HRMS calcd for $C_{14}H_{18}O_3S$ *m/z* 266.0977, observed 266.0972.

*5.3.8. Octyl 2′,5′-dimethoxythiocinnamate (**36**)*

It was obtained as colorless oil in 93% yield. IR (Thin film): 1665, 1607, 1496, 1464, 1288, 1222, 1140, 1031 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, *J* = 7.37 Hz, CH_3), 1.28–1.39 (10H, m, 5× CH_2), 1.63 (2H, m, SCH_2CH_2), 2.99 (2H, t, *J* = 7.3 Hz, SCH_2CH_2), 3.83 (6H, s, 2× OCH_3), 6.71 (1H, d, *J* = 15.7 Hz, C–2H), 6.80–7.0 (3H, m, Ar–H), 7.86 (1H, d, *J* = 15.8 Hz, C–1H) ^{13}C NMR (75.5 MHz, $CDCl_3$): 13.9 (CH_3), 22.5 (C-7″), 28.8, 29.0 and 29.5 (C-2″, C-3″, C-4″, C-5″, C-6″), 31.6 (C-1″), 55.9 (2× OCH_3), 112.4 (C-2), 113.26 and 117.3 (C-3′ and C-4′), 123.63 (C-1′), 125.86 (C-6′), 135.4 (C-1), 153.4 (C-2′ and C-5′) and 191.04 (C=O); HRMS calcd for $C_{19}H_{28}O_3S$ *m/z* 336.1759, observed *m/z* 336.1762.

*5.3.9. Benzyl 2′,5′-dimethoxythiocinnamate (**37**)*

It was obtained as light green oil in 98% yield. IR (thin film): 1665, 1607, 1496, 1464, 1288, 1222, 1140, 1031 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.76 (6H, s, 2× OCH_3), 4.22 (2H, s, CH_2), 6.73–7.31 (9H, m, aromatic protons and C–2H) and 7.92 (1H, d, *J* = 15.8, C–1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): 33.12 (CH_2), 55.69 and 55.98 (2× OCH_3), 112.44, 113.0 and 1117.5 (C-3′, C-4′ and C-6′), 123.48 (C-1′), 123.4, 125.2, 127.1, 128. 5 and 128.8 (C-2″, C-3″, C-4″, C-5″, C-6″ and C-2), 136.2 (C-1), 137.78 (C-1″), 153.5 (C-2′ and C-5′), 189.3 (CO); HRMS calcd for $C_{18}H_{18}O_3S$ *m/z* 314.0977, observed 314.0974.

*5.3.10. Ethyl 3′,4′,5′-trimethoxydihydrothiocinnamate (**42**)*

It was obtained as colorless oil in 98% yield. IR (thin film): 1688, 1590, 1455, 1346, 1239, 1129, 982, 755 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.99 (3H, t, *J* = 7.41 Hz, SCH_2CH_3), 2.59–2.67 (4H, m, 2× CH_2), 3.02 (2H, q, *J* = 7.38 Hz, SCH_2CH_3), 3.58 (9H, s, 3× OCH_3), 6.15 (2H, s, aromatic protons); ^{13}C NMR (75.5 MHz, $CDCl_3$): 16.88 (CH_3), 33.9 (C-2), 47.7 (C-1), 58.2 (3× OCH_3), 62.9 (SCH_2), 107.5 (C-2′ and C-6′), 127.6 (C-1′) 138.06 (C-3′ and 5′), 155.36 C-4′) and 200.7 (CO), HRMS calcd for $C_{14}H_{20}O_4S$ *m/z* 284.1082, observed 284.1080.

*5.3.11. Octyl 3′,4′,5′-trimethoxydihydrothiocinnamate (**43**)*

It was obtained as colorless oil in 98% IR (thin film): 1688, 1591, 1463, 1345, 1239, 1130, 981, 757 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, *J* = 7.35 Hz, CH_3), 1.27–1.55 (12H, m, C-2″H, C-3″H, C-4″H, C-5″H, C-6″ and C-7″H), 2.87–2.89 (6H, m, C-1″H, C–1H, C–2H), 3.8 (9H, s, 3× OCH_3), and 6.4 (2H, s, aromatic protons); ^{13}C NMR (75.5 MHz, $CDCl_3$): 13.9 (CH_3), 28.6, 28.8, 28.9, 28.98, 29.4 and 29.5 (C-2″, C-3″, C-4″, C-5″, C-6″, C-7″), 31.7 (C-2), 45.4 (C-1), 55.9 (3× OCH_3), 60.6 (SCH_2), 105.2 (C-2′ and C-6′), 127.6 (C-1′) 135.7 (C-4′), 153.0 (C-3′ and C-5′) and 198.4 (CO); HRMS calcd for $C_{20}H_{32}O_4S$ *m/z* 368.1941, observed *m/z* 368.1940.

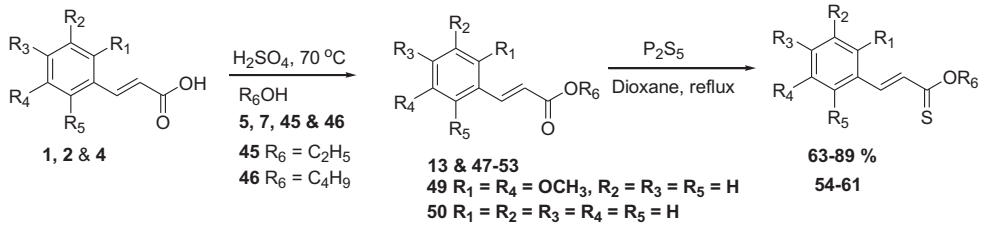
*5.3.12. Benzyl 3′,4′,5′-trimethoxydihydrothiocinnamate (**44**)*

It was obtained as light green oil in 98% yield. IR (thin film): 1687, 1590, 1458, 1346, 1239, 1129, 928, 755 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.87–2.89 (4H, m, C–1H and C–2H), 3.80 (9H, m, 3× OCH_3), 4.1 (2H, s, SCH_2), 6.37 (2H, s, C-2′ and C-6′), 7.23–7.25 (5H, m, aromatic protons); ^{13}CNMR (75.5 MHz, $CDCl_3$): 31.6 (C-2), 45.1 (C-1), 55.9 (3× OCH_3), 60.6 (SCH_2), 105.35 (C-2′ and C-6′), 127.1 (C-4″), 128.51 and 128.65 (C-2″, C-6″, C-3″ and C-5″), 135.6 (C-4′), 143.31 (C-1″), 153.1 (C-3′ and C-5′) and 197.5 (CO); HRMS calcd for $C_{19}H_{22}O_4S$ *m/z* 346.1240, observed *m/z* 346.1244.

*5.4. Synthesis of the thionocinnamates **54**–**61***

The thionocinnamates **54**[1]–**61** were prepared by the sulfonation of their corresponding oxygenated cinnamates **13** and **47**–**53**, [10,34] which in turn were prepared from the corresponding commercially available cinnamic acids and alcohols using P_2S_5 with little modification (Scheme 5) to the literature reported procedure [35]. All the eight cinnamates (**13** and **47**–**53**) were freshly recrystallized prior to their use, and the solvent dioxane was freshly dried and distilled. A mixture of the oxygenated cinnamate **13**/**47**–**53** (100 mg) and P_2S_5 (1.5 equivalent) was refluxed in anhydrous dioxane (10 mL), the progress of the reaction was monitored by TLC. After completion of the reaction, the crude reaction mixture was poured into ice-cold water and extracted with ethyl acetate (3 × 30 mL). The combined organic layer was dried over sodium sulfate and evaporated in *vacuo,* and the residual oil was chromatographed on a silica gel column using petroleum ether. All the novel thionocinnamate **54**–**61** were fully and unambiguously identified on the basis of their spectral (IR, ^1H NMR, ^{13}C NMR spectra and HRMS) analysis. It may be mentioned that though the compounds **58** [35,36] and **54** [37] are mentioned in the literature, but neither of these literature reports have the spectral data of the compounds for comparison, one of them does not have even the correct structure. For compound **54**, no IR, UV, ^{13}C NMR, MS or HRMS, or elemental analysis, or R_f (TLC) values are given, only the

[1] The thionocinnamate **54** (ETMTC) was prepared by using the natural product **47** (ETMC) isolated from *Piper longum.*



Starting Oxygenated Cinnamate	Product Thiono-cinnamate	R_1	R_2	R_3	R_4	R_5	R_6	% Yield
47[10,†]	**54**	H	OCH_3	OCH_3	OCH_3	H	C_2H_5	63
48[10]	**55**	H	OCH_3	OCH_3	OCH_3	H	C_3H_7	69
49[30]	**56**	OCH_3	H	H	OCH_3	H	C_2H_5	78
13[*]	**57**	OCH_3	H	H	OCH_3	H	C_3H_7	79
51[10]	**58**	H	H	H	H	H	C_2H_5	86
52[10]	**59**	H	H	H	H	H	C_3H_7	88
50[10]	**60**	H	H	H	H	H	$CH(CH_3)_2$	88
53[10]	**61**	H	H	H	H	H	C_4H_9	89

[†]The natural product isolated from *Piper longum* was used
*We have prepared **13** as a novel compound (Scheme 1)

Scheme 5.

^{1}H NMR spectrum is reported, that too is not correct, e.g. the two aromatic protons are reported as a singlet at δ 1.13 ppm. Further, no detailed method of preparation or characterization or any cross reference is given. So we strongly suspect that this is not the correct compound reported by a pharmacy group, and the publication is in an obscure non-stream chemistry journal which is not well circulated [37]. There is no other report in the literature on the compound **54** and we have synthesized completely and unambiguously characterized novel compound, viz the most active thionocinnamate **54**. Though there are two literature references [35,36] for the compound **58**, but no spectral data is given for comparison in any of them.

*5.4.1. Ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**)*

It was obtained as an orange solid having melting point 90–91 °C in 63% yield. IR (KBr): 1633 (C═S), 1580, 1464, 1421, 1287, 1126, 1005 cm^{-1} ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.93 (3H, t, J = 7.4 Hz, OCH_2CH_3), 3.81, 3.84 and 3.90 (9H, s, 3× OCH_3), 4.53 (2H, q, J = 6.5 Hz, OCH_2CH_3), 6.73 (2H, s, C–2′H and C–6′H), 6.94 (1H, d, J = 15.6 Hz, C–2H), and 7.60 (1H, d, J = 15.6, C–1H). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 11.02 (OCH_2CH_3), 56.58 (2× OCH_3), 61.36 (OCH_3), 74.10 (OCH_2CH_3), 106.05 (C-2′ and C-6′), 128.72 (C-2), 130.53 (C-1′), 131.20 (C-4′), 140.70 (C-1), 153.86 (C-3′ and C-5′), and 210.73 (C═S). HRMS Calcd for $C_{14}H_{19}O_4S$ $[M + H]^+$ 283.3819, found 283.3820.

*5.4.2. Propyl 3′,4′,5′-trimethoxythionocinnamate (**55**)*

It was obtained as an orange red viscous oil in 69% yield. IR (Thin film): 1635 (C═S), 1580, 1464, 1421, 1287, 1126, 1005 cm^{-1} ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.07 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.91 (2H, m, $OCH_2CH_2CH_3$), 3.82, 3.84 and 3.89 (9H, s, 3× OCH_3), 4.53 (2H, t, J = 6.5 Hz, $OCH_2CH_2CH_3$), 6.70 (2H, s, C-2′H and C-6′H), 6.92 (1H, d, J = 15.6 Hz, C–2H), and 7.61 (1H, d, J = 15.6, C–1H). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 11.04 ($OCH_2CH_2CH_3$), 22.14 ($OCH_2CH_2CH_3$), 56.60 (2× OCH_3), 61.33 (OCH_3), 74.0 ($OCH_2CH_2CH_3$), 106.0 (C-2′ and C-6′), 128.73 (C-2), 130.57 (C-1′), 131.16 (C-4′), 140.72 (C-1), 153.88 (C-3′ and C-5′), and 210.70 (C═S). HRMS Calcd for $C_{15}H_{21}O_4S$ $[M + H]^+$ 297.3819, found 297.3820.

*5.4.3. Ethyl 2′,5′-dimethoxythionocinnamate (**56**)*

It was obtained as an orange red viscous oil in 78% yield. IR (Thin film): 1663 (C═S), 1595, 1496, 1464, 1340, 1278, 1120, 1040 cm^{-1} ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47 (3H, t, J = 6.6 Hz, OCH_2CH_3), 3.78 and 3.85 (6H, s, 2× OCH_3), 4.63 (2H, q, J = 6.5 Hz, OCH_2CH_3), 6.91 (1H, d, J = 14.9 Hz, C–2H), 7.03–7.08 (3H, C–3′H, C–4′H and C–6′H) and 8.02 (1H, d, J = 15.9 Hz, C–1H); ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 14.21 (OCH_2CH_3), 56.22 and 56.53 (2× OCH_3), 68.24 (OCH_2CH_3), 112.91 (C-6′), 113.13 (C-4′), 117.92 (C-3′), 124.61 (C-2), 130.12 (C-1′), 135.51 (C-1), 153.52 (C-2′), 154.01 (C-5′) and 211.2 (C═S). HRMS: Calcd for $C_{13}H_{17}O_3S$ $[M + H]^+$ 253.0820, found 253.0818.

*5.4.4. Propyl 2′,5′-dimethoxythionocinnamate (**57**)*

It was obtained as an orange red viscous oil in 79% yield. IR (Thin film): 1664 (C═S), 1605, 1496, 1462, 1277, 1222, 1175, 1048 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05 (3H, t, J = 7.4 Hz, $OCH_2CH_2CH_3$), 1.90 (2H, m, $OCH_2CH_2CH_3$), 3.80 and 3.85 (6H, s, 2× OCH_3), 4.53 (2H, t, J = 6.6 Hz, $OCH_2CH_2CH_3$), 6.90 (1H, d, J = 14.7 Hz, C–2H), 7.04–7.09 (3H, m, C–3′H, C–5′H and C–6′H) and 8.02 (1H, d, J = 15.9, C–1H); ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 18.21 ($OCH_2CH_2CH_3$), 29.4 ($OCH_2CH_2CH_3$), 63.82 (2× OCH_3), 81.22 ($OCH_2CH_2CH_3$), 120.13 (C-2), 120.31 (C-6′), 125.26 (C-4′), 131.82 (C-1′), 137.31 (C-3′), 142.90 (C-1), 160.81 (C-2′), 161.24 (C-5′) and 218.62 (C═S); HRMS: Calcd for $C_{14}H_{19}O_3S$ $[M + H]^+$ 267.1055, found 267.1055.

*5.4.5. Ethyl thionocinnamate (**58**)*

It was obtained as an orange red oil in 86% yield. IR (Thin film): 1613 (C═S), 1447, 1373, 1328, 1289, 1257, 1185, 1093 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (3H, t, J = 6.14 Hz, OCH_2CH_3), 5.76 (2H, q, J = 6.24 Hz, OCH_2CH_3), 7.0 (1H, d, J = 15.74 Hz, C–2H), 7.35–7.52 (5H, m, C–2′H, C–3′H, C–4′H, C–5′H and C–6′H) and 7.63 (1H, d, J = 15.75, C–1H); ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 21.71 (OCH_2CH_3), 75.21 (OCH_2CH_3), 128.7 (C-2), 129.3 (C-2′ and C-6′), 130 (C-4′), 130.57 (C-3′ and C-5′), 135.24 (C-1′), 140.53 (C-1) and 209.9 (C═S). HRMS: Calcd for $C_{11}H_{13}OS$ $[M + H]^+$ 193.0609, found 193.0610.

*5.4.6. Propyl thionocinnamate (**59**)*

It was obtained as orange red oil in 88% yield. IR (thin film): 1613 (C═S), 1450, 1202, 1170, 979 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.9 (2H, m, $OCH_2CH_2CH_3$), 4.5 (2H, t, J = 6.5 Hz, $OCH_2CH_2CH_3$), 7.04 (1H, d, J = 15.8 Hz, C–2H), 7.30–7.54 (5H, m, C–2′H, C–3′H, C–4′H, C–5′H and C–6′H) and 7.68 (1H, d, J = 15.8 Hz, C–1H). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 10.23 ($OCH_2CH_2CH_3$), 21.4 ($OCH_2CH_2CH_3$), 73.2 ($OCH_2CH_2CH_3$), 128.0 (C-

2), 128.5 (C-2′ and C-6′), 128.7 (C-4′), 129.9 (C-3′ and C-5′), 134.4 (C-1′), 139.95 (C-1) and 210.2 (C=S). HRMS: Calcd for $C_{12}H_{15}OS$ $[M + H]^+$, 207.0844 found. 207.0845.

5.4.7. Isopropyl thionocinnamate (**60**)

It was obtained as orange red viscous oil in 88% yield. IR (thin film): 1613 (C=S), 1447, 1257, 1093, 971, 752 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ 1.41 [6H, d, $J = 6.14$ Hz, $CH(CH_3)_2$], 5.76 (1H, m, $CH(CH_3)_2$), 7.0 (1H, d, $J = 15.8$ Hz, C−2H), 7.35−7.52 (5H, m, C−2′H, C−3′H, C−4′H, C−5′H and C−6′H) and 7.63 (1H, d, $J = 15.8$ Hz, C−1H). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 21.71 ($CH(CH_3)_2$), 75.21 ($CH(CH_3)_2$), 128.7 (C-2), 129.3 (C-4′), 130.06 (C-3′ and C-5′), 130.57 (C-2′ and C-6′), 135.24 (C-1′), 140.53 (C-1) and 209.9 (C=S). HRMS: Calcd for $C_{12}H_{15}OS$ $[M + H]^+$ 207.0844, found 207.0843.

5.4.8. Butyl thionocinnamate (**61**)

It was obtained as orange red oil in 89% yield. IR (thin film): 1656 (C=S), 1618, 1452, 1215, 1040, 756 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$): δ 1.02 (3H, t, $J = 7.2$ Hz, $OCH_2CH_2CH_2CH_3$), 1.52 (2H, m, $OCH_2CH_2CH_2CH_3$), 1.83 (2H, m, $OCH_2CH_2CH_2CH_3$), 4.61 (2H, t, $J = 6.2$ Hz, $OCH_2CH_2CH_2CH_3$), 7.02 (1H, d, $J = 15.8$ Hz, C−2H), 7.3−7.5 (5H, m, C−2′H, C−3′H, C−4′H, C−5′H and C−6′H) and 7.67 (1H, d, $J = 15.8$ Hz, C−1H). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ 13.12 ($OCH_2CH_2CH_2CH_3$), 18.74 ($OCH_2CH_2CH_2CH_3$), 27.75 ($OCH_2CH_2CH_2CH_3$), 71.34 ($OCH_2CH_2CH_2CH_3$), 127.72 (C-2), 128.35 (C-4′), 128.41 (C-3′ and C-5′), 129.62 (C-2′ and C-6′) 134.97 (C-1′), 139.62 (C-1) and 209.9 (C=S). HRMS: Calcd for $C_{13}H_{17}OS$ $[M + H]^+$ 221.3305, found 221.303.

5.5. Cell culture

Primary endothelial cells were isolated from human umbilical cord by mild trypsinization [10]. The cells were grown in M-199 medium supplemented with 15% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, endothelial cell growth factor (50 μg/mL) and heparin (5 U/mL) in 5% CO_2 in humidified water jacketed incubator. The purity of endothelial cells was determined by FACS analysis of E-selectin expression.

5.6. Cell viability assay

The cytotoxicity of ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**) and other analogs was analyzed by using trypan blue exclusion test as described [21], and was further confirmed by colorimetric MTT assay as described [10]. Briefly, endothelial cells were plated onto 96-microwell plates and allowed to attach overnight. Next day, the cells were treated either with DMSO (0.25%) or tested compounds for 24 h followed incubation with 100 μl of MTT (5 mg/mL in PBS) for additional 4 h. Finally cells were washed with PBS, and incubated with 100 μl DMSO to dissolve water insoluble MTT-formazan crystals. Absorbance was recorded at 570 nm in an ELISA reader (BIO RAD, Model 680, and USA). All experiments were performed at least 3 times in triplicate.

5.7. Cell ELISA for measurement of ICAM-1, VCAM-1 and E-selectin

Expression of ICAM-1, VCAM-1 and E-selectin was quantified by whole cell-ELISA [10]. Briefly, endothelial cells were pre-incubated with or without the tested compounds at desired concentrations for 2 h followed by induction with TNF-α (10 ng/mL) or LPS (1 μg/mL) for 16 h for ICAM-1 and VCAM-1 expression and 4 h for E-selectin expression. After incubation, the cells were fixed immediately within the plate with 1.0% glutaraldehyde. Nonspecific protein binding was blocked subsequently using non-fat dry milk (3.0% in PBS). Cells were incubated overnight at 4 °C with primary antibodies for ICAM-1, VCAM-1 and or E-selectin (Santa Cruz, USA). Cells were washed with PBS and incubated with peroxidase-conjugated goat anti-mouse secondary Abs. After a further wash with PBS, cells were incubated with peroxidase substrate (*o*-phenylenediamine dihydrochloride 40 mg/100 mL in citrate phosphate buffer, pH 4.5). The color development reaction was stopped by the addition of 2 N sulfuric acid and absorbance at 490 nm was measured using an automated microplate reader (680 Bio-Rad, USA).

5.8. Determination of IC_{50}

The percentage inhibitions of each compound at its various log concentrations were measured and plotted graphically from three independent experiments. From the graph plotted, the concentration at which a compound showed 50% inhibition was taken as its IC_{50} value.

5.9. Flow cytometry analysis

The cell surface expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells was further confirmed by flow cytometry [10]. Briefly, endothelial cells were incubated with or without ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**) for 2 h followed by TNF-α (10 ng/mL). E-selectin was measured at 4 h whereas ICAM-1 and VCAM-1 expression was measured 16 h after TNF-α treatment. The cells were washed with PBS and dislodged, following which they were incubated with anti-ICAM-1, anti-VCAM-1, anti-E-selectin or control IgG antibody (1.0 μg/10^6 cells, 30 min, 4 °C). After incubation, the cells were washed with PBS and then stained with FITC-conjugated goat anti-mouse IgG for 30 min at 4 °C. Finally, the cells were fixed with 1.0% paraformaldehyde and analyzed for the expression of cell adhesion molecules using a flow cytometer (FACS Vantage, Becton & Dickinson, USA). For each sample, 20,000 events were acquired. Analysis was carried out by using Cell Quest Software (Becton Dickinson, USA). The auto-fluorescence intensity was subtracted from treated conditions and mean fluorescence intensity was estimated from three independent experiments and bar diagrams were plotted.

5.10. Neutrophils isolation

Neutrophils were isolated from peripheral blood of healthy individuals [19]. Briefly, blood was collected in heparin solution (20 U/mL) and erythrocytes were removed by sedimentation against 6% dextrin solution. Plasma rich white blood cells were layered over ficoll-hypaque solution followed by centrifugation (300 g for 20 min, 20 °C). The top saline layer and the ficoll-hypaque layer were aspirated leaving the neutrophils/RBC pellet. The residual red blood cells were removed by hypotonic lysis. Isolated cells were washed with PBS and resuspended in PBS containing 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at a final concentration of 6×10^5 cells/mL. This procedure usually resulted in approximately 95% neutrophils and the cell viability was more than 95% as detected by trypan blue exclusion test.

5.11. Neutrophils adhesion assay

Neutrophils adhesion assay was performed under static conditions as described previously [19]. Endothelial cells were plated to confluence in 96-well culture plates and were incubated with or without ethyl 3′,4′,5′-trimethoxythionocinnamate (**54**) for 2 h followed by induction with TNF-α (10 ng/mL) for 6 h. After washing, the endothelial monolayer was incubated with neutrophils (6×10^4/well) for 1 h at 37 °C. The non-adherent neutrophils were

removed by washing the wells with PBS and neutrophils bound to endothelial cells were assayed by adding a substrate solution consisting of *o*-phenylenediamine dihydrochloride (40 mg/100 mL in citrate phosphate buffer, pH 4.5), 0.1% cetitrimethyl ammonium bromide and 3-amino-1,2,4 triazole (1 mM). The absorbance was determined at 490 nm using an automated microplate reader (Spectramax 190, Molecular Devices, USA).

6. Statistical analysis

Results are given as means ± SD. Independent two-tailed Student's *t* test was performed. Differences were considered statistically significant for $p < 0.05$. The statistical analysis was performed using Microcal Origin software (ver 3.0; Microcal Software Inc, Northampton, MA and Cell Quest Software, Becton–Dickinson, USA).

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