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Abstract

Based on the importance of the previous fluorinated and/or hydroxylated chalcones studies, thirty-six compounds were designed as phenyl or hydroxyphenyl bearing fluoro, trifluoromethyl or trifluoromethoxy phenyl propenones and synthesized by applying modified Claisen-Schmidt condensation reaction as a single step. Inhibitory effects of the synthesized compounds on ROS production stimulated by LPS in RAW 264.7 macrophage were evaluated. Structure-activity relationship (SAR) study revealed that the compounds possessing *para*-hydroxyphenyl group combined with *meta*-fluoro or *meta*-trifluoromethyl phenyl group, and *meta*/*para*-hydroxyphenyl group combined with *ortho*-trifluoromethoxyphenyl group have an essential role in inhibiting the LPS- stimulated ROS production in RAW 264.7 macrophages. The most significant inhibitory effect on LPS-stimulated ROS production in RAW 264.7 macrophages was observed in compound **30** that possessed *para*-hydroxyphenyl group along with *ortho*-trifluoromethoxyphenyl group.

Keywords: fluorinated and hydroxylated chalcones, inhibition of LPS-stimulated ROS production, SAR study

Partially reduced oxygen species such as hydroxyl radical, peroxyl radical, superoxide radical, and hydrogen peroxide, formed during normal cellular metabolism are known as reactive oxygen species (ROS) and are responsible for both beneficial and harmful activities. Beneficial effects of ROS occur at low or moderate concentrations and include cellular signaling, defense against infectious agents, and the induction of a mitogenic response. On the other hand, overproduction of ROS, known as oxidative stress, can cause significant damage to biological systems which can further contribute to the development of various degenerative conditions such as DNA mutation, cancer, aging, neurodegenerative disorders, cell death, and inflammation [1, 2]. Macrophages are considered to have important roles in host immune defense systems during infection and disease development. Activation of macrophages by stimuli such as bacterial lipopolysaccharide (LPS) [3, 4], induces the production of ROS which is the key to the progression of many inflammatory diseases. Reactive nitrogen species (RNS) such as peroxynitrite are formed by the rapid combination of nitric oxide (NO) with superoxide radical. The RNS, in turn, induces nitrosative stress that further contributes to the inflammatory burden of ROS [4]. Recently, malvidin, which is included in the polyphenols of red wine, has been shown to inhibit ROS production triggered by LPS in RAW 264.7 macrophages and the concentration that inhibits 50% of LPS-induced ROS production has been found to be 9.0± 0.8 μM [5]. In addition, the *in-vitro* anti-oxidant activity [6] along with anti-inflammatory [7], antibacterial [8], and neuroprotective [9] activities of malvidin have been reported. Although diverse chemical compounds have been reported to combat the oxidative as well as inflammatory conditions, potent compounds with negligible unwanted effects are still lacking. Therefore, additional pharmacological agents that aim to control the excessive production of ROS and possess minimal side effects could be considered as one of the promising treatment options for several pathological conditions.

Chalcone (1,3-diphenylpropenone) is one of the major constituents obtained from natural products and is an important precursor for synthetic manipulations. Chalcones, along with their synthetic analogues, demonstrate diverse biological activities such as antibacterial, antifungal, antimalarial [10, 11], antituberculosis [11], antifilarial [12], antileishmanial [13], antioxidant, antidyslipidemia [14], neuroprotective [15], anticancer [16], modulation of P-glycoprotein-mediated multidrug resistance [17], and also have anti-inflammatory effects [18]. Previously, our research group has synthesized various chalcone derivatives **(Fig. 1)** by replacing two phenyl rings of a chalcone with heteroaromatic rings, and reported those to

have anti-inflammatory properties through considerable dual COX/5-LOX inhibitory activity [19] as well as anti-angiogenic and anti-tumor activity [20-22]. In addition, thienyl/furanyl-hydroxyphenylpropenones, previously synthesized by our research group, as chalcone derivatives, displayed significant inhibition of ROS production stimulated by LPS in RAW 264.7 macrophages [23].

From various studies, it has been found that most of the phenolic and polyphenolic derivatives show antioxidant [24], anti-inflammatory [25], anti-angiogenic, and antiproliferative properties [26, 27]. Further, hydroxylated chalcones found in nature as well as their synthetic analogues are reported to have several pharmacological activities such as antiprotozoal, anti-plasmodium activity [28], antifungal activity [29], xanthine oxidase inhibitors, radical scavengers activity [30, 31], antibacterial [32, 33], anti-diabetic and anti-obesity activity [34] including analgesic and anti-inflammatory activity [35]. Similarly, a large bulky group such as halogen atoms tend to occupy most of the deeper pockets as well as the reacting regions of the molecular targets, thereby inducing either antagonistic or agonist activity. Recently, in drug discovery optimization, many studies are performed by introducing fluorine atoms (-F, $-CF_3$, or $-OCF_3$) in the structure because of their capacity to enhance potency, bioavailability, cell permeability, and also chemical and metabolic stability by increasing molecules' lipophilicity and acting as hydrogen bond acceptors [36]. Likewise, several studies about the fluorinated chalcones have found in them significant antiproliferative [37], anti-invasive activity [38], antiperoxidation, 5-lipoxygenase inhibitory activity [39], MAO-B inhibitory activity, analgesic, antioxidant, and anti-inflammatory activity [40]. From these various reports exhibiting the importance of the phenolic group together with the introduction of the fluorine atom has inspired us to integrate the following functional groups in the chalcones that we have synthesized as shown in **Fig. 2.**

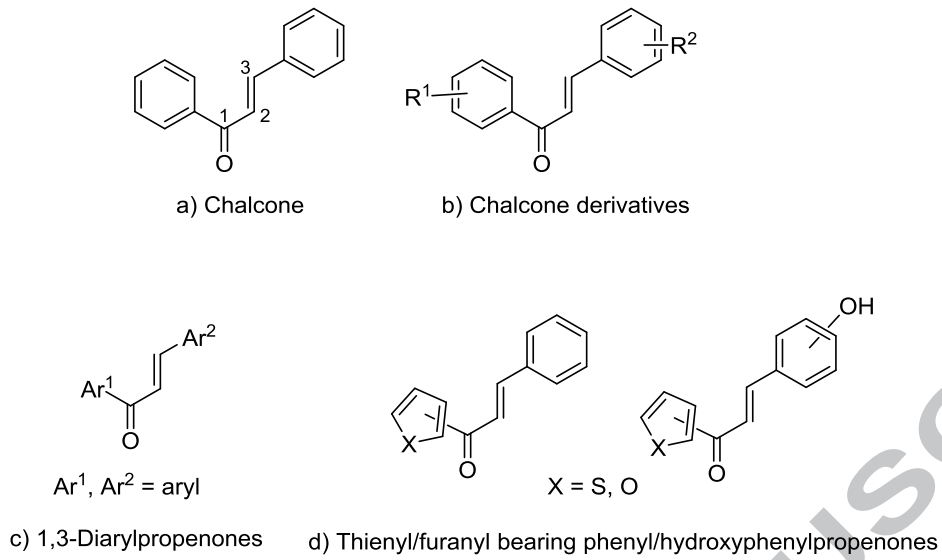


Fig. 1. Structures of a) chalcone, b) chalcone derivatives, c) 1,3-diarylpropenones, and d) thienyl/furanyl bearing phenyl/hydroxyphenyl propenones

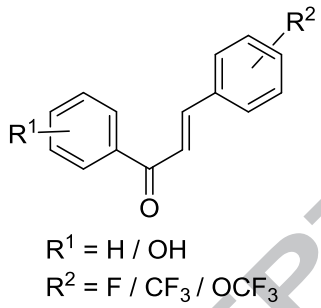
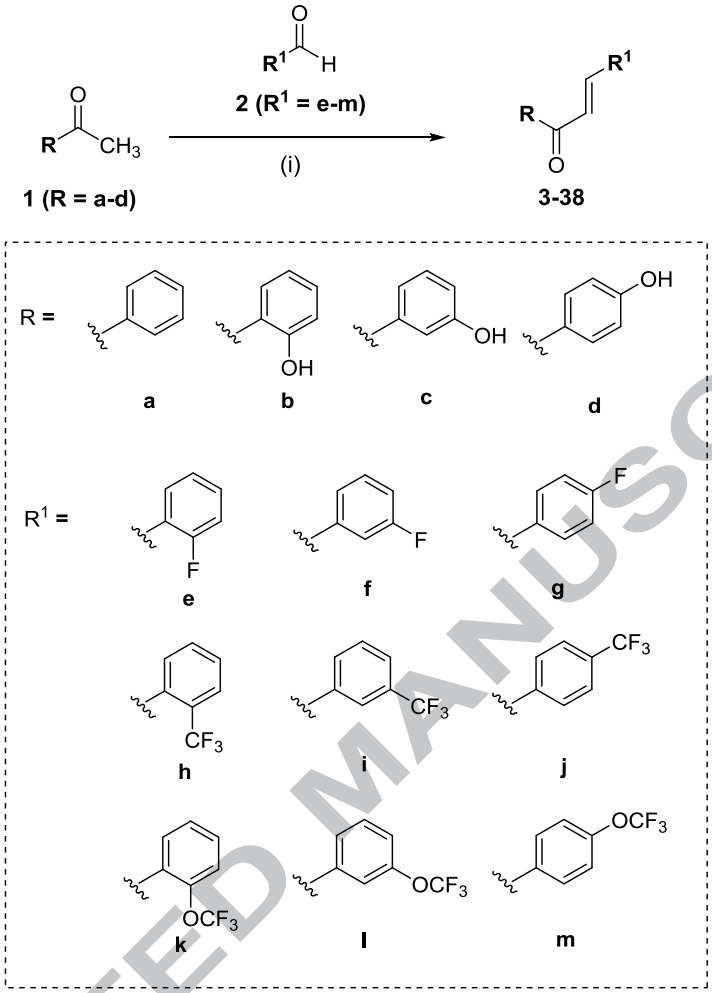


Fig. 2. Design of phenyl/hydroxyphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones as fluorinated and hydroxylated chalcones

In this following study, we have consistently designed and prepared thirty-six phenyl or hydroxyphenyl bearing fluoro, trifluoromethyl, or trifluoromethoxy phenyl propenones as fluorinated or both fluorinated and hydroxylated chalcones. Fluorinated functionalities on 3-phenyl moiety and *ortho*, *meta*, or *para*-phenols of chalcone were prepared in order to investigate the inhibitory effects on ROS production stimulated by LPS in RAW 264.7 macrophages. A Structure-activity relationship (SAR) study was conducted with respect to *ortho, meta*, and *para* substitution along with fluorinated phenyl groups on chalcones.

Firstly, we designed and synthesized phenyl or hydroxyphenyl bearing fluoro, trifluoromethyl, or trifluoromethoxy phenyl propenones (**3-38**) via the single step modified Claisen-Schmidt condensation reaction [41, 42] using appropriate aryl methyl ketone **1**(R= **a-d**) and aryl aldehyde **2** (R^1= **e-m**) as summarized in **Scheme 1.** Compounds **3-38** were obtained through base catalyzed reactions without any protection of hydroxyl group that involves the addition of 50% aqueous KOH (1.0 mL) to a solution of aryl methyl ketone **1** (1.0 mmol) and aryl aldehyde **2** (1.2 mmol) in ethanol (3 mL). The yields were 32.1 to 82.7%.

Total thirty-six compounds (**3-38**) were synthesized as shown in **Fig. 3**. Among them, compounds **3-14** contained phenyl, *ortho*-, *meta*-, or *para*-hydroxyphenyl moiety along with *ortho*-, *meta*-, or *para*-fluorophenyl moiety. Compounds **15-26** contained phenyl, *ortho*-, *meta*-, or *para*-hydroxyphenyl moiety along with *ortho*-, *meta*-, or *para*-trifluoromethylphenyl moiety. Similarly, compounds **27-38** contained phenyl, *ortho*-, *meta*-, or *para*-hydroxyphenyl moiety along with *ortho*-, *meta*-, or *para*-trifluoromethoxyphenyl moiety. The SAR was determined according to the position of hydroxyl, fluoro, trifluoromethyl, or trifluoromethoxy group, as well as the presence or absence of hydroxyl group in the phenyl ring of chalcone.



Scheme 1. Schematic representation for the synthesis of phenyl/hydroxylphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones **3-38** as fluorinated and hydroxylated chalcones. Reagents and conditions: (i) 50% aq. KOH (9.0 equiv., 1mL), EtOH (3 mL), 1-3 h, 25°C, 32.1 to 82.7% yield.

Fig. 3. Synthesized phenyl/hydroxyphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones **3-38**

The prepared compounds were evaluated for inhibitory effect on ROS production stimulated by LPS in RAW 264.7 macrophage as shown in **Table 1.**

Table 1. Inhibitory activities of compounds **3-38** on ROS production stimulated by LPS in RAW 264.7 macrophages

Compounds	ROS Inhibition (IC_{50}, µM)	Toxicity	Compounds	ROS Inhibition (IC_{50}, µM)	Toxicity
3	>10	N	**22**	1.44 ± 0.56	N
4	>10	N	**23**	>10	N
5	>10	N	**24**	>10	N
6	>10	N	**25**	>10	N
7	>10	N	**26**	>10	N
8	>10	N	**27**	>10	N
9	>10	N	**28**	ND	Toxic*
10	9.15 ± 1.77	N	**29**	1.61 ± 1.06	N
11	>10	N	**30**	1.34 ± 0.44	N
12	>10	N	**31**	>10	N
13	>10	N	**32**	ND	Toxic*
14	>10	N	**33**	>10	N
15	>10	N	**34**	>10	N
16	ND	Toxic*	**35**	>10	N
17	>10	N	**36**	>10	N
18	>10	N	**37**	>10	N
19	>10	N	**38**	>10	N
20	ND	Toxic*	Malvidin [5]	9.0 ± 0.8	
21	ND	Toxic*			

N: Non-toxic, ND: Not Determined, *Less than 90% cell viability at 1 µM, **Each data represents mean ± S.D. from three different experiments performed in triplicate

Amongst the first twelve synthesized compounds **3-14**, that had the basic skeleton of hydroxyphenyl-fluorophenylpropenone either with a fluorine substituent at the *ortho*, *meta*, or *para* position on the 3-phenyl ring of chalcone (**3, 7, 11**), or with both hydroxyl and fluorine substituents at the *ortho*, *meta*, or *para* position on the 1, and 3-phenyl ring of chalcone (**4-6, 8-10, 12-14**), compound **10** significantly inhibited LPS-stimulated ROS production in RAW 264.7 macrophages (9.15 µM of IC_{50}). This demonstrated that a hydroxyl group at the *para* position along with a fluorine atom at the *meta* position is essential for inhibiting the LPS-stimulated ROS production in RAW 264.7 macrophages. Among the next twelve synthesized compounds **15-26**, that had the basic skeleton of hydroxyphenyl-trifluoromethylphenyl propenone either with a trifluoromethyl substituent at the *ortho*, *meta*, or *para* position on the 3-phenyl ring of chalcone (**15**, **19, 23**), or with both hydroxyl and trifluoromethyl substituents at the *ortho*, *meta*, or *para* position on the 1, and 3-phenyl ring of chalcone (**16-18, 20-22, 24-26**), compound **22** significantly inhibited ROS production stimulated by LPS in RAW 264.7 macrophages (1.44 µM of IC_{50}). This also showed that a hydroxyl group at the *para* position along with a trifluoromethyl group at the *meta* position is essential for inhibiting the LPS-stimulated ROS production in RAW 264.7 macrophages. Among the last twelve synthesized compounds **27-38**, having the basic skeleton of hydroxyphenyl-trifluoromethoxyphenyl propenone either with a trifluoromethoxy substituent at the *ortho*, *meta*, or *para* position on the 3-phenyl ring of chalcone (**27, 31, 35**), or with both hydroxyl and trifluoromethoxy substituents at the *ortho*, *meta*, or *para* position on the 1, and 3-phenyl ring of chalcone (**28-30, 32-34, 36-38**), compound **29** and **30** significantly inhibited LPS-stimulated ROS production in RAW 264.7 macrophages (1.61 and 1.34 µM of $IC_{50,}$ respectively). This demonstrated that a hydroxyl group at the *meta* or *para* position along with a trifluoromethoxy group at the *ortho* position is essential for inhibiting the LPS-stimulated ROS production in RAW 264.7 macrophages. Among the thirty-six synthesized compounds, five compounds **16**, **20**, **21**, **28**, and **32** showed toxicity, which possessed *ortho*-hydroxyphenyl moiety along with trifluoromethyl or trifluoromethoxy phenyl moiety. It was observed that 3-phenyl bearing fluoro, trifluoromethyl, trifluoromethoxy phenyl propenones without a hydroxyl substituent on 1-phenyl moiety had no significant inhibition of LPS-stimulated ROS production in RAW 264.7 macrophages. Analyzing the structure-activity relationship revealed that the compounds possessing *para*-hydroxyphenyl moiety along with *meta*-fluoro or *meta*-trifluoromethyl phenyl group, and *meta*- or *para*-hydroxyphenyl group along with *ortho*-trifluoromethoxyphenyl group showed significant inhibition of LPS-

stimulated ROS production in RAW 264.7 macrophages. It was also observed that most of the *ortho*-hydroxyphenyl moiety along with trifluoromethyl, or trifluoromethoxyphenyl moiety possessed toxic properties. Therefore, it can be concluded that a hydroxyl group at the *para* position of the 1-phenyl ring along with a fluoro or trifluoromethyl group at the *meta* position, or a trifluoromethoxy group at the *ortho* position of the 3-phenyl ring of chalcone has an essential role in inhibiting LPS-stimulated ROS production in RAW 264.7 macrophages, which is explained in **Fig. 4.**

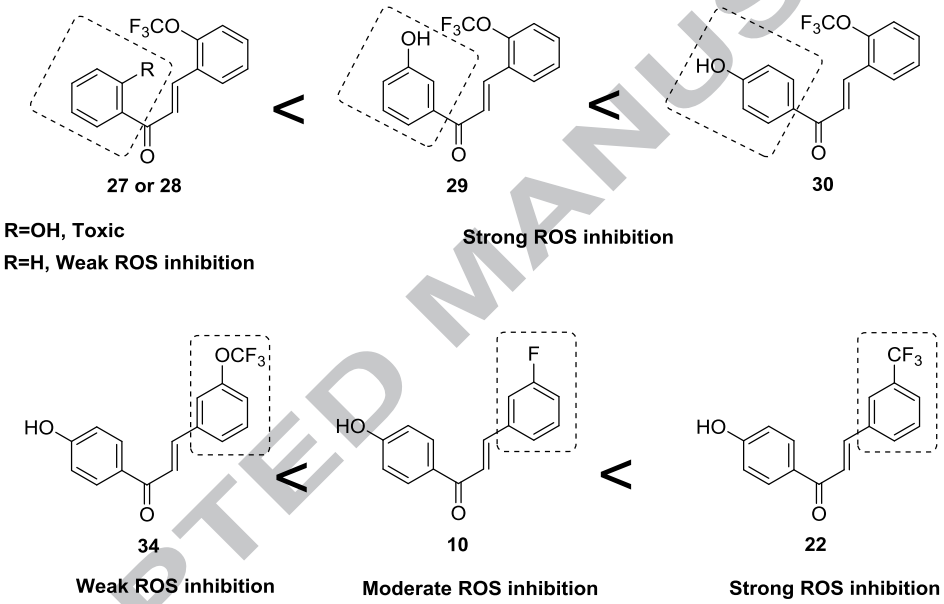


Fig. 4. Favorable substitution order on chalcone for the inhibition of ROS production stimulated by LPS in RAW 264.7 macrophages from SAR study

In summary, systematical design and synthesis of the thirty-six phenyl or hydroxyphenyl bearing fluoro, trifluoromethyl, or trifluoromethoxy phenyl propenones were performed using *Claisen-Schmidt* condensation reaction. Inhibitory effects of these compounds on LPS-stimulated ROS production in RAW 264.7 macrophages were examined. A concrete structure-activity relationship was observed according to the positions of substitutions of hydroxyl and fluorinated groups. The most significant inhibitory effect on LPS-stimulated ROS production in RAW 264.7 macrophages was exhibited by compound **30** that possessed *para*-hydroxyphenyl moiety as well as *ortho*-trifluoromethoxyphenyl moiety.

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Figure captions

Fig. 1. Structures of a) chalcone, b) chalcone derivatives, c) 1,3-diarylpropenones, and d) thienyl/furanyl bearing phenyl/hydroxyphenyl propenones

Fig. 2. Design of phenyl/hydroxyphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones as fluorinated and hydroxylated chalcones

Fig. 3. Synthesized phenyl/hydroxyphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones **3-38**

Fig. 4. Favorable substitution order on chalcone for inhibition of ROS production stimulated by LPS in RAW 264.7 macrophages for SAR study

Scheme caption

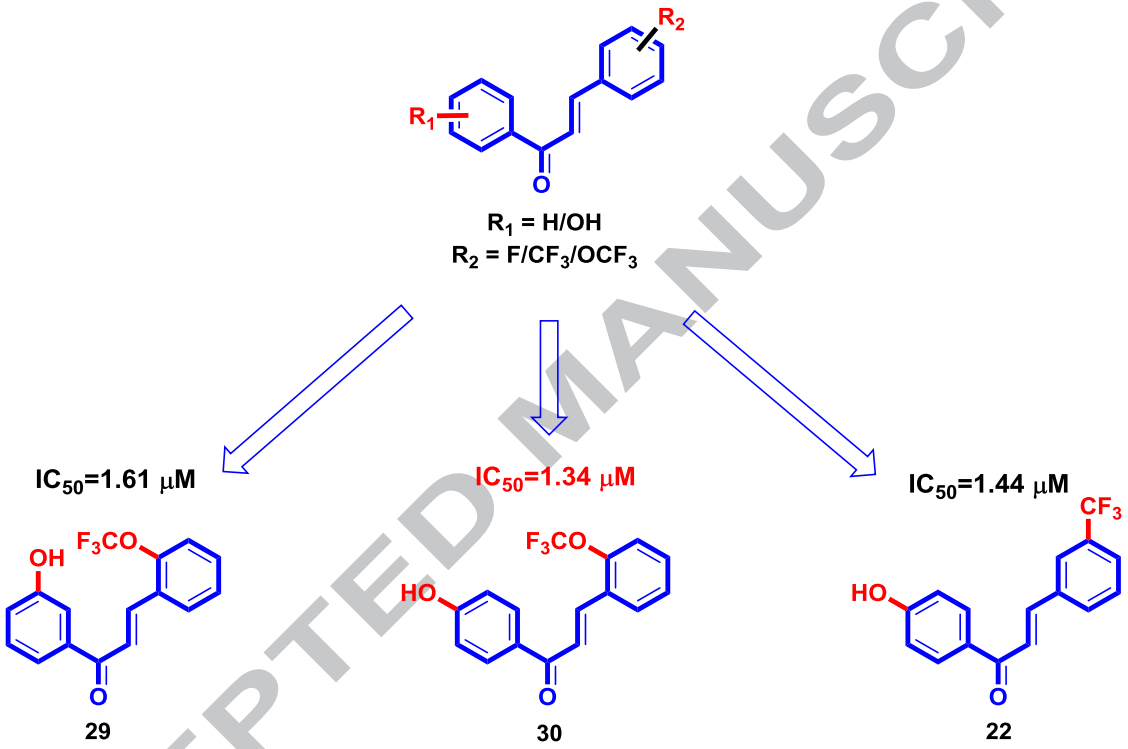
Scheme 1. Schematic representation for the synthesis of phenyl/hydroxylphenyl bearing fluoro/trifluoromethyl/trifluoromethoxy phenyl propenones **3-38** as fluorinated and hydroxylated chalcones. Reagents and conditions: (i) 50% aq. KOH (9.0 equiv., 1mL), EtOH (3 mL), 1-3 h, 25°C, 32.1 to 82.7% yield.

Table caption

Table 1. Inhibitory activities of compounds **3-38** on ROS production stimulated by LPS in RAW 264.7 macrophages

Graphical abstract

Graphical Abstract



Strong inhibition of LPS-stimulated ROS production in RAW 264.7 macrophage

Highlights

- Designed and synthesized new fluorinated and hydroxylated chalcones.
- Inhibitory effects on LPS-stimulated ROS production in RAW 264.7 macrophage were evaluated.
- SAR showed *p*-OH along with *m*-F or CF_3, and *m*- or *p*-OH along with *o*-OCF_3 has an essential role for inhibition.
- Compounds **30** showed the most potent inhibitory effect.