Accepted Manuscript

Nitric oxide inhibitory activity and antioxidant evaluations of 2-benzoyl-6-benzylidenecyclohexanone analogues, a novel series of curcuminoid and diarylpentanoid derivatives

Sze Wei Leong, Siti Munirah Mohd Faudzi, Faridah Abas, Mohd Fadhlizil Fasihi Mohd Aluwi, Kamal Rullah, Lam Kok Wai, Mohd Nazri Abdul Bahari, Syahida Ahmad, Chau Ling Tham, Khozirah Shaari, Nordin H. Lajis

PII: S0960-894X(15)00530-2
DOI: http://dx.doi.org/10.1016/j.bmcl.2015.05.056
Reference: BMCL 22746

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 22 January 2015
Revised Date: 11 May 2015
Accepted Date: 22 May 2015

Please cite this article as: Leong, S.W., Faudzi, S.M.M., Abas, F., Aluwi, M.F.F., Rullah, K., Wai, L.K., Bahari, M.N.A., Ahmad, S., Tham, C.L., Shaari, K., Lajis, N.H., Nitric oxide inhibitory activity and antioxidant evaluations of 2-benzoyl-6-benzylidenecyclohexanone analogues, a novel series of curcuminoid and diarylpentanoid derivatives, *Bioorganic & Medicinal Chemistry Letters* (2015), doi: http://dx.doi.org/10.1016/j.bmcl.2015.05.056

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

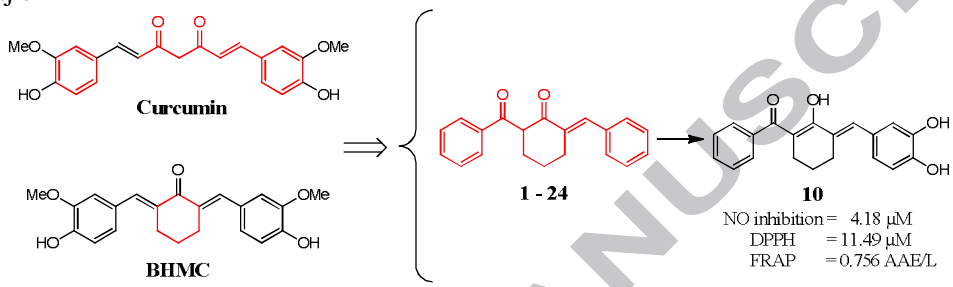
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Nitric oxide inhibitory activity and antioxidant evaluations of 2-benzoyl-6-benzylidenecyclohexanone analogues, a novel series of curcuminoid and diarylpentanoid derivatives

Leave this area blank for abstract info.

Sze Wei Leong[a], Siti Munirah Mohd Faudzi[a,f], Faridah Abas[a,b*], Mohd Fadhlizil Fasihi Mohd Aluwi[c], Kamal Rullah[c], Lam Kok Wai[c], Mohd Nazri Abdul Bahari[d], Syahida Ahmad[d], Chau Ling Tham[e], Khozirah Shaari[a,f], Nordin H. Lajis[a,*]



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Nitric oxide inhibitory activity and antioxidant evaluations of 2-benzoyl-6-benzylidenecyclohexanone analogues, a novel series of curcuminoid and diarylpentanoid derivatives

Sze Wei Leong[a], Siti Munirah Mohd Faudzi[a,f], Faridah Abas[a,b*], Mohd Fadhlizil Fasihi Mohd Aluwi[c], Kamal Rullah[c], Lam Kok Wai[c], Mohd Nazri Abdul Bahari[d], Syahida Ahmad[d], Chau Ling Tham[e], Khozirah Shaari[a,f], Nordin H. Lajis[a,*]

[a]*Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*
[b]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*
[c]*Drug and Herbal Research Centre Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abd. Aziz, 50300 Kuala Lumpur, Malaysia.*
[d]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*
[e]*Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[f]*Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Anti-inflammatory
Antioxidant
2-benzoyl-6-benzylidenecyclohexanone
RAW 264.7
Stability

ABSTRACT

A series of twenty-four 2-benzoyl-6-benzylidenecyclohexanone analogues were synthesized and evaluated for their nitric oxide inhibition and antioxidant activity. Six compounds (**3**, **8**, **10**, **17**, **18** and **19**) were found to exhibit significant NO inhibitory activity in LPS/IFN-induced RAW 264.7 macrophages, of which compound **10** demonstrated the highest activity with the IC_{50} value of 4.2 ± 0.2 μM. Furthermore, two compounds (**10** and **17**) displayed antioxidant activity upon both the DPPH scavenging and FRAP analyses. However, none of the 2-benzoyl-6-benzylidenecyclohexanone analogues significantly scavenged NO radical. Structure-activity comparison suggested that 3,4-dihydroxylphenyl ring is crucial for bioactivities of the 2-benzoyl-6-benzylidenecyclohexanone analogues. The results from this study and the reports from previous studies indicated that compound **10** could be a candidate for further investigation on its potential as a new anti-inflammatory agent.

2014 Elsevier Ltd. All rights reserved.

Curcumin is a well-known chemical constituent abundantly found in turmeric (*Curcuma longa*), which has received enormous attention by numerous researchers for its broad range of pharmacological properties with extremely mild side effects.[1-7] Curcumin has been reported to exhibit incredible antioxidant, anti-inflammatory and anti-cancer properties upon their excellent superoxide and free radical inhibition, pro-inflammatory cytokines and enzymes suppression, and cancer cells apoptosis induction.[8-11] Curcumin has also been shown to acts as antibacterial and anti-malarial agents based on its cytotoxic effect on several bacteria and parasites, such as *Helicobacter pylori* and *Plasmodium falciparum*.[12,13] However, the practical use of curcumin is limited by its poor absorbability and stability, resulting in low oral bioavailability, which therefore diminishes its usefulness in clinical trials.[14] On this account, synthetic modifications of curcumin have been widely employed and intensively studied in many laboratories in order to develop a molecule with improved bioactivities and stability.

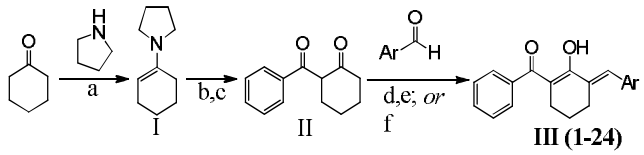
Diarylpentanoids, a 5-carbon spacer series resulted from the synthetic modification of curcumin which has gained increasing attention due to its curcumin-like medicinal properties. Diarylpentanoids have been shown to exhibit remarkable anti-inflammatory activity based on their positive inhibitions on a wide range of pro-inflammatory cytokines, enzymes and mediators such as nitric oxide (NO), prostaglandins (PGs), tumor necrosis factor alpha [TNF-α, interleukins, lipoxygenase (LOX) and cyclooxygenases].[15-18] They are also found to display great anti-cancer properties as they significantly inhibited the growth of various cancer cell lines including breast, lung, colon, pancreas and prostate cancer cells, through their anti-proliferative and anti-angiogenic activities.[19-23] Unlike curcumin, diarylpentanoids are chemically stable at physiological pH and metabolically stable in rat liver microsomes, which make them the most impactful candidates among curcuminoids deserving further intensive investigations towards developing novel bioactive molecules with good stability and bioavailability Previously, we have shown that α,β-unsaturated β-diketone and

*Corresponding authors. Tel.: +603 8947 1491
E-mail addresses: faridah_abas@upm.edu.my *(Faridah Abas);*
nordinlajis@gmail.com *(N.H.Lajis)*

ACCEPTED MANUSCRIPT

cyclohexanone fragments are crucial for diarylpentanoids' bioactivities in different studies.[15,26] In continuing our effort on the search of new anti-inflammatory agent, we have now synthesized a series of 2-benzoyl-6-benzylidenecyclohexanone analogues, a novel family of diarylpentanoid which incorporated the integration of α,β-unsaturated β-diketone and cyclohexanone moieties.

Scheme 1. General synthetic steps for compounds 1-24[x]



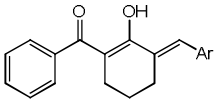
[x]Reagents and conditions: (a) *p*-toluene-sulphonic acid, toluene, reflux (2h); (b) benzoic anhydride, RT (24h); (c) H_2O, reflux (0.5h); (d) B_2O_3, tributyl borate, n-butylamine, ethyl acetate, 70°C (3 h); (e) HCl, reflux (0.5 h); (f) benzaldehyde, acetic acid, H_2SO_4, RT (overnight).

As represented in Scheme 1, compounds **1-24** (**III**) were successfully synthesized through a series of reactions, which involved benzoylation of cyclohexanone and aldol condensation with aryl aldehydes. The benzoylation of cyclohexanone was accomplished by Stork enamine acylation as prescribed by Kaiho et.al. in 1989.[27] Accordingly, cyclohexanone was first reacted with pyrrolidine to afford *N*-(1-cyclohexenyl)pyrrolidine (**I**), an essential enamine intermediate for benzoylation. The reaction was performed in Dean-Stark apparatus in order to eliminate the water side-product, which would otherwise hydrolyze the *N*-(1-cyclohexenyl)pyrrolidine back to its precursors. The *N*-(1-cyclohexenyl)pyrrolidine was then reacted with benzoic anhydride to obtain the corresponding crude product of 2-benzoylcyclohexanones (**II**). Subsequent purification with flash chromatography yielded 2-benzoylcyclohexanone with conversion rate of 30-40%. The purified 2-benzoylcyclohexanone was subsequently reacted with a range of different aryl aldehydes to achieve the desired compounds **III** through aldol condensation.[28] In this step, acidic condition was the method of choice since it can accomplish higher yields of the desired products, and in a shorter time as compared to that conducted in basic condition. The synthesized compounds were purified through flash chromatography and characterized by ^{1}H-NMR, ^{13}C-NMR, and high-resolution electron impact-mass spectrometry. The ^{1}H-NMR spectra of all 2-benzoyl-6-benzylidenecyclohexanone analogues exhibited an intense and sharp singlet at 16-17 ppm, indicative of the chelated hydroxyl groups and that these compounds are more stable in the keto-enol rather than in their diketo form. All purified compounds used for the NO inhibition and antioxidant evaluations were of 95 to 99% purity based on their respective HPLC profiles.

The synthesized compounds were evaluated for their nitric oxide (NO) inhibitory activity in IFN-γ/LPS-stimulated RAW 264.7 macrophages.[29] Nitric oxide is an important mediator involved in the inflammatory process. It is biosynthesized endogenously from L-arginine, oxygen and NADPH, and catalyzed by various nitric oxide synthase (NOS) enzymes. Appropriate level of NO produced is responsible for body's defense mechanism against abnormalities.[30] However, the overproduction of NO may promote tissue injuries through cell lipids peroxidation and deoxyribonucleic acid (DNA) mutation, which may eventually lead to several chronic degenerative diseases including arthritis, multiple sclerosis, ulcerative colitis, neurodegenerative diseases, cardiovascular disorder, and cancers.[31-35] Therefore, pharmacological intervention of NO production is a rapid and promising strategy in the search for new potent drugs against inflammatory related degenarative diseases.

The preliminary evaluation for NO inhibitory activity was conducted on LPS/IFN induced RAW 264.7 cells at 50 μM test concentration. The results showed that six compounds were significantly suppressed the NO production with the percentage of inhibition at greater than 50%. The six bioactive compounds were then subjected to IC_{50} determination and the values obtained were compared to that of the positive control, curcumin. MTT assay was subsequently carried out to confirm that NO inhibition is not due to cytotoxicity. The NO inhibitory effect of 2-benzoyl-6-benzylidenecyclohexanone analogues are presented in Table 1.

Table 1. NO Suppression activity and cytotoxicity of compounds **1-24** on RAW 264.7 Cells.



Compounds	Ar	NO inhibition at 50 μM (%) ± S.E.M	NO inhibition IC_{50} (μM) ± S.E.M	Cytotoxicity IC_{50} (μM) ± S.E.M
Curcumin	-	99.3 ± 0.2	14.7 ± 0.2	28.8 ± 0.8
1	phenyl	5.3 ± 2.7	ND	ND
2	2-methoxyphenyl	6.9 ± 5.7	ND	ND
3	3-methoxyphenyl	71.6 ± 0.9	32.1 ± 1.0	>100
4	4-methoxyphenyl	30.8 ± 5.1	ND	ND
5	2-chlorophenyl	21.8 ± 2.8	ND	ND
6	3-chlorophenyl	34.5 ± 5.4	ND	ND
7	4-chlorophenyl	2.0 ± 1.7	ND	ND
8	3-hydroxyphenyl	80.0 ± 1.5	23.3 ± 0.6	87.8 ± 1.9
9	4-hydroxyphenyl	25.6 ± 2.9	ND	ND
10	3,4-dihydroxyphenyl	86.0 ± 2.0	4.2 ± 0.2	53.9 ± 2.9
11	naphthalen-1-yl	15.0 ± 2.2	ND	ND
12	naphthalen-2-yl	9.3 ± 4.9	ND	ND
13	2,3-dimethoxyphenyl	29.8 ± 7.4	ND	ND
14	2,5-dimethoxyphenyl	3.7 ± 1.8	ND	ND
15	3,4-dimethoxyphenyl	8.2 ± 2.2	ND	ND
16	3,4,5-trimethoxyphenyl	15.8 ± 2.4	ND	ND
17	4-hydroxy-3-methoxyphenyl	69.0 ± 2.6	35.3 ± 1.4	98.5 ± 0.4
18	3-chloro-4-hydroxyphenyl	73.4 ± 4.9	22.7 ± 0.5	61.6 ± 3.3
19	3-bromo-4-hydroxyphenyl	83.5 ± 3.0	15.2 ± 0.8	56.4 ± 1.6

ACCEPTED MANUSCRIPT

20	5-methylfuran-2-yl	15.6 ± 4.7	ND	ND
21	thiophen-2-yl	8.2 ± 3.5	ND	ND
22	5-methylthiophen-2-yl	7.6 ± 4.0	ND	ND
23	3-fluorophenyl	8.8 ± 5.0	ND	ND
24	2,3,4-trimethoxyphenyl	9.1 ± 4.0	ND	ND

ND = Not determine

Two compounds (**10** and **19**) were found to exhibit strong NO inhibitory activity, giving IC_{50} values of 4.2 and 15.2 μM, respectively. Meanwhile, four other compounds (**3**, **8**, **17** and **18**) displayed moderate NO inhibitory activity with the IC_{50} values of 22 to 47 μM. Based on these results, it appears that phenolic group is essential for NO suppression activity as five out of six active compounds bearing at least one hydroxyl group on their phenyl ring. Besides, it has also been found that the substitution pattern of functional groups does affect the NO inhibitory activity. The comparison of *meta*- and *para*- hydroxylated compounds (compounds **8** and **9**, respectively) shows that substitution at *meta*-position is preferable as indicated by a relatively stronger NO inhibitory activity of **8** over compound **9**. A similar trend was also noted in the comparison of compounds **2-4**, of which compound **3**, a *meta*-methoxylated analog exhibited much stronger activity than the other two analogs. These results are consistent with the results in previous studies, which found that *meta*-oxygenated phenyl ring is favored for anti-inflammatory and anti-cancer properties' enhancement.[36,37] Surprisingly, apart from oxygenated functionalities, the activity enhancement effect of *meta*-substitution was also been observed in *meta*-halogenated analogs. This can be seen in the comparison of compounds **9**, **18** and **19**, of which compounds **18** and **19**, the *meta*-halogenated analogs displayed much stronger NO inhibitory effect than its meta-unsubstituted analog **9**. All these observations suggested that the presence of functionalities at *meta*-position of phenyl ring could improve the anti-inflammatory potential.

More importantly, 3,4-dihydroxylphenyl moiety appeared to be the most significant contributing factor as compound **10**, a 3,4-dihydroxylphenyl containing 2-benzoyl-6-benzylidenecyclohexanone, which exhibited the strongest NO inhibitory activity in a dose-dependent manner (see Fig. 1A and Fig. 1B). It displayed the IC_{50} value of 4.2 μM, which is 4-fold stronger than curcumin (14.7 μM). Subsequent MTT assay has proven that the strong activity of compound **10** was not due to its cytotoxic effect (see Fig. 1C). This finding is consistent with our previous finding that a catechol moiety is important for anti-inflammatory activity.[26]

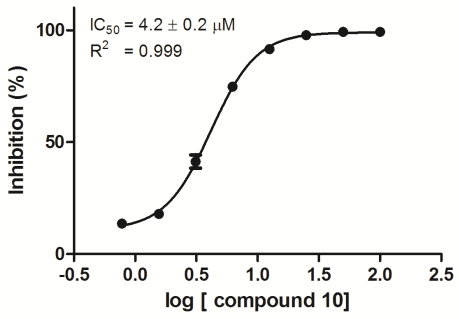


Fig. 1A. Dose-response curve of compound **10** on NO inhibition in LPS/IFN-induced RAW 264.7 macrophages.

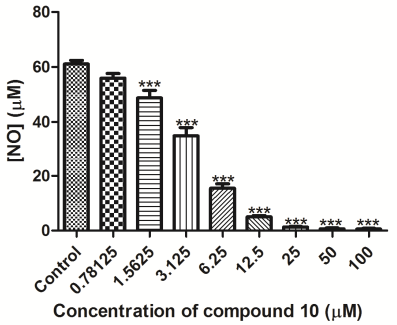


Fig. 1B. NO secretion of LPS/IFN induced RAW 264.7 cells upon treatment with compound **10** at different concentration.

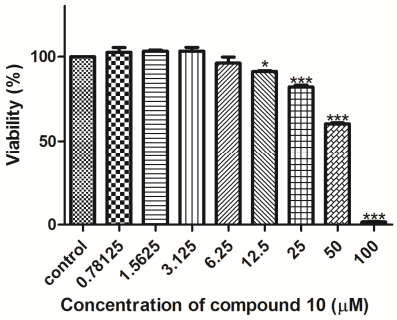


Fig. 1C. Cell viability of LPS/IFN-induced RAW 264.7 macrophage cells upon treatment with compound **10** at different concentration using MTT assay.

Similar observation has also been made in other bioactive scaffolds such as flavonoids, diarylheptanoids, and aurones, which further support the anti-inflammatory potential of catechol containing compound.[38-40] Interestingly, a previous study by Chiang and co-workers has revealed that the activity enhancing effect of catechol moiety could be boosted by a neighboring α,β-unsaturated carbonyl moiety.[41] On account of these and the results from this study, it is thus reasonable to suggest that compound **10** could be a potent drug candidate that deserve a further in-depth *in vitro* and *in vivo* analyses as it contains both catechol and α,β-unsaturated carbonyl moieties. Unlike our previous study on 1,5-diphenyl-1,3-pentenediones, the electron density does not significantly affects the activity of the present compound series as all high (**13-16**) and low (**5-7**) electron density compounds are poor in NO inhibitory activity. Therefore, it is conceivable to conclude that hydrogen bonding capacity is the only responsible factor for anti-inflammatory activity of 2-benzoyl-6-benzylidenecyclohexanone analogues.

All synthesized compounds were also evaluated for their antioxidant properties through NO scavenging, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and ferric reducing antioxidant power (FRAP) assays.[42] The results obtained were analyzed and presented in Table 2.

ACCEPTED MANUSCRIPT

Table 2. Direct NO scavenging, DPPH scavenging and FRAP activity of compounds **1-24.**

Compounds	Direct NO Scavenging activity at 500 µM (%)) ± S.E.M	DPPH radical scavenging activity		FRAP (mmole AAE/L)
		at 200 µM (%)) ± S.E.M	IC_{50} (µM) ± S.E.M	
Ascorbic acid	2.6 ± 1.2	-	-	-
BHT	6.5 ± 2.6	-	-	0.798
Trolox	12.4 ± 1.1	-	-	0.395
Curcumin	88.3 ± 2.1	84.2 ± 1.6	23.6 ± 1.1	0.803
1	17.9 ± 2.1	14.0 ± 2.4	ND	0.015
2	27.3 ± 3.3	13.6 ± 2.3	ND	0.024
3	28.3 ± 1.1	21.8 ± 1.5	ND	0.018
4	31.7 ± 2.3	20.1 ± 1.7	ND	0.032
5	17.8 ± 3.0	17.1 ± 3.2	ND	0.011
6	15.8 ± 1.0	25.0 ± 1.2	ND	0.008
7	30.9 ± 1.0	14.0 ± 1.7	ND	0.001
8	6.5 ± 2.9	17.3 ± 1.8	ND	0.042
9	3.5 ± 2.0	15.5 ± 3.2	ND	0.080
10	30.5 ± 4.2	85.1 ± 1.3	11.5 ± 0.4	0.756
11	14.8 ± 2.6	17.8 ± 1.8	ND	0.015
12	21.0 ± 3.0	11.7 ± 1.4	ND	0.073
13	9.9 ± 3.1	9.9 ± 1.2	ND	0.034
14	13.8 ± 1.5	5.1 ± 4.2	ND	0.178
15	10.9 ± 1.3	7.7 ± 1.2	ND	0.237
16	3.4 ± 1.8	8.3 ± 2.9	ND	0.038
17	7.4 ± 2.4	67.5 ± 1.1	47.6 ± 2.1	0.379
18	27.6 ± 1.2	15.4 ± 2.3	ND	0.079
19	31.7 ± 2.0	18.0 ± 2.0	ND	0.086
20	4.9 ± 2.1	27.0 ± 1.9	ND	0.177
21	3.3 ± 2.1	21.8 ± 2.1	ND	0.177
22	1.6 ± 0.6	18.4 ± 2.0	ND	0.140
23	1.9 ± 0.9	23.4 ± 2.3	ND	0.018
24	9.3 ± 3.7	8.0 ± 3.4	ND	0.131

ND = Not determine

Two compounds, **10** and **17** were found to display significant activity in both DPPH radical scavenging and FRAP assays, of which compound **10** demonstrated the highest antioxidant potential. It exhibited a 2-fold higher activity than curcumin in DPPH scavenging assay and comparable activity to butylated hydroxytoluene (BHT) in FRAP analysis. The dose-dependent response of these compounds and the respective positive controls in DPPH and FRAP assays are shown in Fig. 2 and Fig. 3, respectively. On the other hand, all 2-benzoyl-6-benzylidenecyclohexanones were found to be inactive towards the direct NO scavenging as none of the compounds significantly inhibited NO radical even at high concentration of 500 µM. This finding is in agreement with the results in our previous study, which found that all mono-carbonyl diarylpentanoids failed to perform NO scavenging effect.

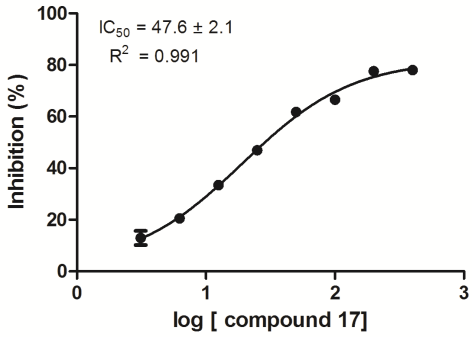


Fig. 2b.DPPH scavenging activity of compound **17** at different concentrations.

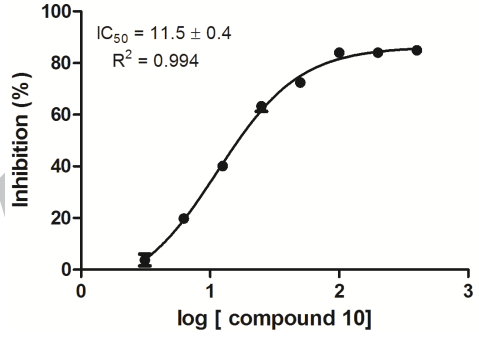


Fig. 2a. DPPH scavenging activity of compounds **10** at different concentration.

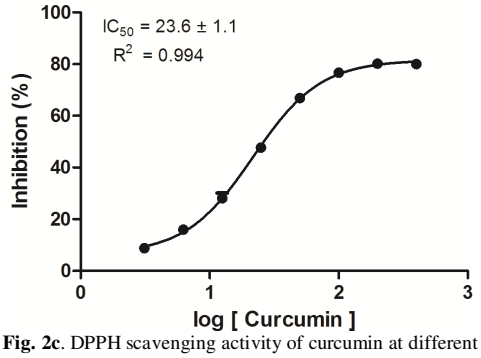


Fig. 2c. DPPH scavenging activity of curcumin at different concentrations.

ACCEPTED MANUSCRIPT

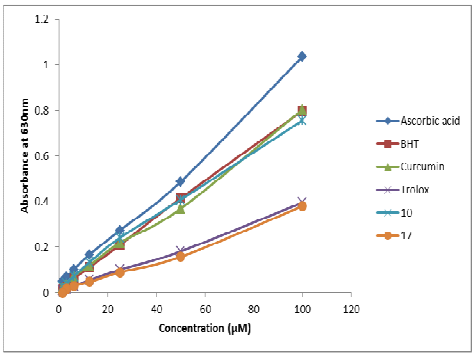


Fig. 3. Ferric-reducing antioxidant power of compounds **10**, **17**, curcumin, and the reference antioxidants (ascorbic acid, BHT and trolox) at different concentrations

The water solubility of Curcumin and compound**10** were further evaluated according to the method described by Colclough et al. with some modifications.[43] Generally, the water solubility test was carried out by introducing DMSO stock solution of test compounds into phosphate buffer (pH 7.4) in the ratio of 1 to 99. After 24 hours of stirring, the concentrations of respective compounds in phosphate buffer were determined by quantitative HPLC analysis. The results from this experiment are presented in Table 3. Compound **10** was found to possess 3-fold better water solubility than curcumin with the concentration of 3.3 µM or 1.1 µg/mL. Further modification or formulation of **10** may be necessary to improve its water solubility since 1.1 µg/mL is categorized as slightly soluble according to the General Notices and Requirements in USP 38 NF 33.[44]

Table 3. Water solubility of curcumin and compound **10.**

Compounds	Solubility	
	µM	µg/mL
Curcumin	1.1	0.4
10	3.3	1.1

Curcumin and compound **10** were also subjected to chemical stability test. The stability of compound **10** at physiological pH was compared with curcumin based on the procedure described by Zhang et al.[45] The test was carried out by observing the ultraviolet spectra changes of targeted compounds for 30 minutes with 5-minutes interval in phosphate buffer (pH 7.4). Fig. 4A and Fig. 4B showed the ultraviolet changes of curcumin and compound **10**, respectively. As shown in Fig. 4, the maximum absorption peaks of curcumin gradually decreased over time. However, no significant changes was observed in the case of compound **10** which indicated that compound **10** is chemically more stable than curcumin *in vitro*. This finding is consistent with the previous results of Zhang et.al, in which showed that mono-carbonyl diarylpentanoid derivatives are chemically more stable than curcumin.

.

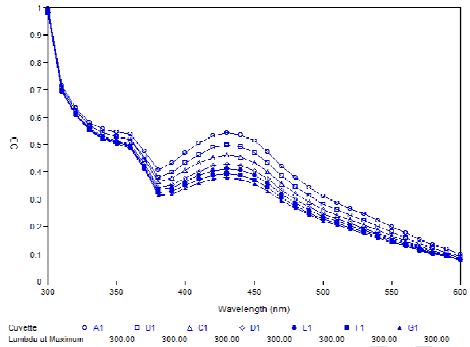


Fig. 4A. Ultraviolet-visible absorption spectra of curcumin.

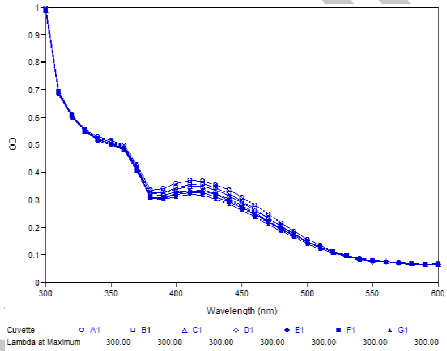


Fig. 4B. Ultraviolet-visible absorption spectra of compound **10**.

To further investigate the potential of compound **10**, *in vitro* metabolic stability analysis was also performed based on the procedure described by Stout et.al.[46] The test was carried out by observing the changes in the concentration of the compound due to the metabolic action of human liver microsome, which are measured at different time points. Fig. 5 depicted the metabolic stability of curcumin and compound **10** in human liver microsome.

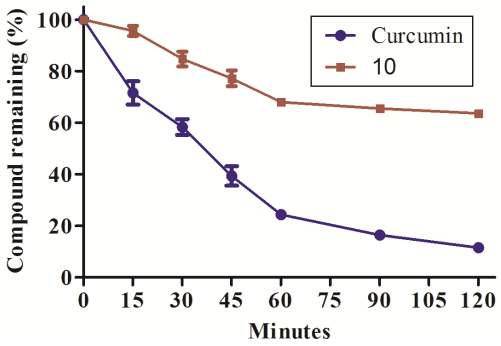


Fig. 5. Metabolic stability of curcumin and compound **10** in human liver microsome.

As shown in Fig. 5, concentrations of both curcumin and compound **10** gradually decreased over the time of the experiment. However, the concentration of curcumin was found to be reduced in much faster rate than that of compound **10,** as less than 20% of curcumin was detected after 120 minutes of metabolism, while more than 60% of compound **10** was still detected upon the same treatment. These observations indicated that compound **10** exhibited much better metabolic stability than curcumin. Taken both chemical and metabolic stability analyses together, we concluded that compound 10 exhibits better *in vitro* stability in comparison with curcumin.

ACCEPTED MANUSCRIPT

In summary, our previous and current findings suggested that 3,4-dihydroxylphenyl is a crucial moiety for bioactivity of diarylpentanoids and might have promising therapeutic potential as anti-inflammatory agents. Based upon this, we concluded that compound **10** could be used as a lead to be further developed into a new anti-inflammatory agent as it exhibited remarkable NO inhibitory and antioxidant activities, and possess excellent stability. However, further mechanistic studies such as iNOS protein and RNA expressions, release and expression of pro-inflammatory mediators, regulatory protein expression in inflammation-related pathways are required to understand NO inhibition mechanism of compound **10** in RAW 264.7 cells.

Acknowledgments

The authors thank the Ministry of Education (MOE) of Malaysia and Universiti Putra Malaysia and for financial support under the project ERGS/1/11/STG/UPM/01/24. NHL also thanks the Scientific Chair Unit, Taibah University for its support. The first author acknowledges the support from the Malaysian Ministry of Science, Technology and Innovation (MOSTI) for scholarship under the National Science Foundation (NSF).

References and notes

1. Naik, S. R.; Thakare, V. N.; Patil, S. R. *Exp. Toxicol. Pathol.* **2011**, *63*, 419.
2. Gupta, S. C.; Sung, B.; Kim, J. H.; Prasad, S.; Li, S.; Aggarwal, B. B. *Mol. Nutr. Food Res.* **2013**, *57*, 1510.
3. Wang, Y.; Lu, Z.; Wu, H.; Lv, F. *Int. J. Food Microbiol.* **2009**, *136*, 71.
4. Reddy, R. C.; Vatsala, P. G.; Keshamouni, V. G.; Padmanaban, G.; Rangarajan, P. N. *Bioche.m Biophys. Res. Commun.* **2005**, *326*, 472.
5. Limtrakul, P.; Lipigorngoson, S.; Namwong, O.; Apisariyakul, A.; Dunn, F. W. *Cancer Lett.* **1997**, *116*, 197.
6. Gururaj, A. E.; Belakavadi, M.; Venkatesh, D. A.; Marme, D.; Salimath, B. P. *Biochem. Biophys. Res. Commun.* **2002**, *297*, 934.
7. Bisht, K.; Wagner, K. H.; Bulmer, A. C. *Toxicology* **2010**, *278*, 88.
8. Rao, C. V. *Adv. Exp. Med. Biol.* **2007**, *595*, 213.
9. Ben, P.; Liu, J.; Lu, C.; Xu, Y.; Xin, Y.; Fu, J.; Huang, H.; Zhang, Z.; Gao, Y.; Luo, L.; Yin, Z. *Int. Immunopharmacol.* **2011**, *11*, 179.
10. Ak, T.; Gulcin, I. *Chem. Biol. Interact.* **2008**, *174*, 27.
11. Wu, S. H.; Hang, L. W.; Yang, J. S.; Chen, H. Y.; Lin, H. Y.; Chiang, J. H.; Lu, C. C.; Yang, J. L.; Lai, T. Y.; Ko, Y. C.; Chung, J. G. *Anticancer Res.* **2010**, *30*, 2125.
12. De, R.; Kundu, P.; Swarnakar, S.; Ramamurthy, T.; Chowdhury, A.; Nair, G. B.; Mukhopadhyay, A. K. *Antimicrob. Agents Chemother.* **2009**, *53*, 1592.
13. Cui, L.; Miao, J. *Antimicrob. Agents Chemother.* **2007**, *51*, 488.
14. Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B. *Mol. Pharm.* **2007**, *4*, 807.
15. Lee, K. H.; Ab Aziz, F. H.; Syahida, A.; Abas, F.; Shaari, K.; Israf, D. A.; Lajis, N. H. *Eur. J. Med. Chem.* **2009**, *44*, 3195.
16. Liang, G.; Li, X.; Chen, L.; Yang, S.; Wu, X.; Studer, E.; Gurley, E.; Hylemon, P. B.; Ye, F.; Li, Y.; Zhou, H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1525.
17. Zhao, C.; Yang, J.; Wang, Y.; Liang, D.; Yang, X.; Li, X.; Wu, J.; Wu, X.; Yang, S.; Liang, G. *Bioorg. Med. Chem.* **2010**, *18*, 2388.
18. Ahmad, W.; Kumolosasi, E.; Jantan, I.; Bukhari, S. N.; Jasamai, M. *Chem. Biol. Drug Des.* **2014**, *83*, 670.
19. Fang, X.; Fang, L.; Gou, S.; Cheng, L. *Bioorg. Med. Chem. lett.* **2013**, *23*, 1297.
20. Nakhjiri, M.; Safavi, M.; Alipour, E.; Emami, S.; Atash, A. F.; Jafari-Zavareh, M.; Ardestani, S. K.; Khoshneviszadeh, M.; Foroumadi, A.; Shafiee, A. *Eur. J. Med. Chem.* **2012**, *50*, 113.
21. Liang, G.; Shao, L.; Wang, Y.; Zhao, C.; Chu, Y.; Xiao, J.; Zhao, Y.; Li, X.; Yang, S. *Bioorg. Med. Chem.* **2009**, *17*, 2623.
22. Wei, X.; Du, Z. Y.; Zheng, X.; Cui, X. X.; Conney, A. H.; Zhang, K. *Eur. J. Med. Chem.* **2012**, *53*, 235.
23. Quincoces Suarez, J. A.; Rando, D. G.; Santos, R. P.; Goncalves, C. P.; Ferreira, E.; de Carvalho, J. E.; Kohn, L.; Maria, D. A.; Faiao-Flores, F.; Michalik, D.; Marcucci, M. C.; Vogel, C. *Bioorg. Med. Chem.* **2010**, *18*, 6275.
24. Singh, R. S.; Das, U.; Dimmock, J. R.; Alcorn, J. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2010**, *878*, 2796.
25. Zhang, Y.; Zhao, C.; He, W.; Wang, Z.; Fang, Q.; Xiao, B.; Liu, Z.; Liang, G.; Yang, S. *Drug Des. Devel. Ther.* **2014**, *8*, 373.
26. Leong, S. W.; Faudzi, S. M.; Abas, F.; Aluwi, M. F.; Rullah, K.; Wai, L. K.; Bahari, M. N.; Ahmad, S.; Tham, C. L.; Shaari, K.; Lajis, N. H. *Molecules* **2014**, *19*, 16058.
27. Kaiho, T.; San-Nohe, K.; Kajiya, S.; Suzuki, T.; Otsuka, K.; Ito, T.; Kamiya, J.; Maruyama, M. *J. Med. Chem.* **1989**, *32*, 351.

General procedure for synthesis of I and II. A catalytic amount of *p*-toluenesulphonic acid was added into a mixture of cyclohexanone (20 mmol) and pyrrolidine (20 mmol) in 30 mL of toluene kept in 100 mL SNRB at room temperature. The mixture was then refluxed on a Dean & Stark apparatus for 2 hours to prepare **I**. Upon completion, 20 mmol of benzoic anhydride in 20 mL of toluene was added dropwise into the reaction solution (**I**) and stirred for 24 hours. Distilled water (10 ml) was then added and further refluxed for 30 min. The resulting reaction mixture was extracted thrice with 3M HCl and once with 20 mL water. The toluene layer was dried over anhydrous magnesium sulphate and concentrated in vacuo to give crude **II**. The crude of **II** was purified by gravitational column chromatography

28. Lim, G. S.; Jung, B. M.; Lee, S. J.; Song, H. H.; Kim, C.; Chang, J. Y. *Chem. Mater.* **2007**, *19*, 460.

General procedure for the synthesis of III (pathway d & e). Boron trioxide (1.5 g) was added into a 50 mL SNRB flask containing 10 mL of ethyl acetate in the presence of 2-benzoylcyclohexanone, and stirred at 70 ^{0}C for 3 hours. Appropriate benzaldehyde (20 mmol) and tributyl borate (20 mmol) were then added into the solution and the mixture was stirred for 30 minutes. Catalytic amount of n-butylamine was added dropwise and stirred for overnight followed by reflux with 10 mL of 1 M HCl for 30 minutes. The reaction mixture was extracted with EA and dried over anhydrous magnesium sulphate. The desired product was purified by gravitational column chromatography.

General procedure for the synthesis of III (pathway f). In a 50 mL SNRB, 2-benzoylcyclohexanone (5 mmol) and appropriate aromatic aldehyde (5 mmol) were dissolved in 30 mL of acetic acid. Catalytic amount of concentrated sulfuric acid was added and reaction mixture was stirred for overnight. The resulting mixture was extracted with EA and washed with 10% sodium bicarbonate solution. The organic layer was then dried over anhydrous magnesium sulphate and evaporated using rotatory evaporator. The target

ACCEPTED MANUSCRIPT

compound was purified by gravitational column chromatography.

2-benzoyl-6-benzylidenecyclohexen-1-ol (1). Yellow amorphous powder; 58.33%; m.p.: 126-127ºC; Mass calculated: 290.1307; Mass found: 290.1324. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.0 Hz, 2 H) 2.50 - 2.57 (m, 2 H) 2.75 - 2.82 (m, 2 H) 7.29 - 7.35 (m, 1 H) 7.38 - 7.49 (m, 7 H) 7.59 (dd, J=7.9, 1.5 Hz, 2 H) 7.78 (s, 1 H) 16.78 (s, 1 H).^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.2, 27.6, 108.4, 127.6, 128.1, 128.2, 128.4, 130.1, 130.6, 132.6, 133.4, 136.3, 138.3, 176.3, 195.0

2-benzoyl-6-(2-methoxybenzylidene)cyclohexen-1-ol (2). Yellow amorphous powder; 54.12%; m.p.: 127-128ºC; Mass calculated: 320.1412; Mass found: 320.1422. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.66 (dt, J=11.4, 5.8 Hz, 2 H) 2.53 (t, J=5.7 Hz, 2 H) 2.70 (t, J=5.8 Hz, 2 H) 3.88 (s, 3 H) 6.93 (d, J=8.2 Hz, 1 H) 6.98 (t, J=7.4 Hz, 1 H) 7.29 - 7.37 (m, 2 H) 7.41 - 7.48 (m, 3 H) 7.54 - 7.61 (m, 2 H) 7.96 (s, 1 H) 16.77 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.7, 27.4, 27.6, 55.5, 108.1, 110.6, 119.9, 125.3, 127.6, 128.1, 129.2, 129.7, 130.2, 130.1, 132.4, 138.5, 158.0, 176.4, 195.5

2-benzoyl-6-(3-methoxybenzylidene)cyclohexen-1-ol (3) . Yellow amorphous powder; 58.75%; m.p.: 95-97ºC; Mass calculated: 320.1412; Mass found: 320.1424. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.67 (quin, J=6.1 Hz, 2 H) 2.51 - 2.56 (m, 2 H) 2.75 - 2.81 (m, 2 H) 3.84 (s, 3 H) 6.88 (dd, J=8.2, 2.0 Hz, 1 H) 6.99 (s, 1 H) 7.05 (d, J=7.6 Hz, 1 H) 7.32 (t, J=7.9 Hz, 1 H) 7.41 - 7.50 (m, 3 H) 7.58 (dd, J=8.0, 1.6 Hz, 2 H) 7.74 (s, 1 H) 16.75 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.2, 27.6, 55.3, 108.5, 113.8, 115.5, 122.6, 127.6, 128.1, 129.3, 130.6, 132.8, 133.2, 137.7, 138.3, 159.5, 176.2, 195.5

2-benzoyl-6-(4-methoxybenzylidene)cyclohexen-1-ol (4). Yellow amorphous powder; 59.01%; m.p.: 120-121ºC; Mass calculated: 320.1412; Mass found: 320.1421. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.0 Hz, 2 H) 2.54 (t, J=6.0 Hz, 2 H) 2.78 (t, J=5.4 Hz, 2 H) 3.85 (s, 3 H) 6.94 (d, J=8.7 Hz, 2 H) 7.40 - 7.47 (m, 5 H) 7.58 (dd, J=7.7, 1.6 Hz, 2 H) 7.74 (s, 1 H) 16.93 (s, 1 H). ^{13}C NMR (126 MHz, CHLOROFORM-d) δ ppm 23.6, 27.1, 27.7, 55.4, 108.0, 113.9, 127.7, 128.1, 129.0, 130.5, 130.6, 131.9, 133.4, 138.3, 159.7, 177.5, 194.3

2-benzoyl-6-(2-chlorobenzylidene)cyclohexen-1-ol (5). Yellow amorphous powder; 66.21%; m.p.: 92-94ºC; Mass calculated: 324.0917; Mass found: 324.0932. ^{1}H NMR (500 MHz $CDCl_3$) δ: 1.67 (quin, J=6.1 Hz, 2 H) 2.54 (t, J=6.0 Hz, 2 H) 2.59 - 2.64 (m, 2 H) 7.23 - 7.30 (m, 2 H) 7.35 (dd, J=7.0, 2.0 Hz, 1 H) 7.41 - 7.50 (m, 4 H) 7.58 (dd, J=8.2, 1.5 Hz, 2 H) 7.85 (s, 1 H) 16.56 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.3, 27.4, 108.7, 126.2, 127.5, 128.1, 129.2, 129.7, 130.1, 130.5, 130.7, 134.2, 134.7, 134.8, 138.4, 174.7, 196.7

2-benzoyl-6-(3-chlorobenzylidene)cyclohexen-1-ol (6). Yellow amorphous powder; 64.93%; m.p.: 97-99ºC; Mass calculated: 324.0917; Mass found: 324.0928. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.0 Hz, 2 H) 2.54 (t, J=6.0 Hz, 2 H) 2.71 - 2.78 (m, 2 H) 7.27 - 7.35 (m, 3 H) 7.40 - 7.51 (m, 4 H) 7.58 (dd, J=8.0, 1.3 Hz, 2 H) 7.67 (s, 1 H) 16.62 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$ $CDCl_3$) δ: 23.5, 27.2, 27.5, 108.7, 127.6, 128.0, 128.2, 128.2, 129.6, 129.6, 130.7, 131.5, 133.8, 134.3, 138.14, 138.2, 175.2, 196.2

2-benzoyl-6-(4-chlorobenzylidene)cyclohexen-1-ol (7). Yellow amorphous powder; 68.32%; m.p.: 129-130ºC; Mass calculated: 324.0917; Mass found: 324.0925. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.1 Hz, 2 H) 2.50 - 2.57 (m, 2 H) 2.70 - 2.76 (m, 2 H) 7.37 (s, 4 H) 7.42 - 7.50 (m, 3 H) 7.58 (dd, J=8.0, 1.3 Hz, 2 H) 7.69 (s, 1 H) 16.69 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.5, 27.2, 27.5, 108.6, 127.6, 128.1, 128.6, 130.7, 131.3, 131.9, 133.1, 134.0, 134.8, 138.2, 175.6, 195.8

2-benzoyl-6-(3-hydroxybenzylidene)cyclohexen-1-ol (8). Yellow amorphous powder; 45.14%; m.p.: 120-121ºC; Mass calculated: 306.1256; Mass found: 306.1258. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.66 (quin, J=6.0 Hz, 2 H) 2.53 (t, J=6.0 Hz, 2 H) 2.76 (t, J=5.5 Hz, 2 H) 5.39 (br. s., 1 H) 6.80 (dd, J=8.0, 2.2 Hz, 1 H) 6.91 (s, 1 H) 7.02 (d, J=7.9 Hz, 1 H) 7.22 - 7.29 (m, 1 H) 7.40 - 7.50 (m, 3 H) 7.58 (d, J=7.3 Hz, 2 H) 7.70 (s, 1 H) 16.71 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.5, 27.2, 27.6, 108.6, 115.4, 116.7, 122.7, 127.6, 128.2, 129.5, 130.7, 132.8, 133.1, 137.8, 138.2, 155.5, 176.2, 195.8

2-benzoyl-6-(4-hydroxybenzylidene)cyclohexen-1-ol (9). Yellow amorphous powder; 43.74%; m.p.: 158-160ºC; Mass calculated: 306.1256; Mass found: 306.1268. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.0 Hz, 2 H) 2.53 (t, J=5.8 Hz, 2 H) 2.77 (t, J=5.5 Hz, 2 H) 6.87 (d, J=8.5 Hz, 2 H) 7.38 (d, J=8.2 Hz, 2 H) 7.41 - 7.49 (m, 3 H) 7.57 (d, J=7.3 Hz, 2 H) 7.71 (s, 1 H) 16.87 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.1, 27.7, 108.1, 115.4, 127.6, 128.1, 129.1, 130.5, 130.6, 132.1, 133.4, 138.2, 155.8, 177.5, 194.4

2-benzoyl-6-(3,4-dihydroxybenzylidene)cyclohexen-1-ol (10). Yellow amorphous powder; 36.45%; m.p.: 212-213ºC; Mass calculated: 322.1205; Mass found: 322.1215. ^{1}H NMR (500 MHz, acetone) δ ppm 1.67 (quin, J=5.6 Hz, 2 H) 2.54 (t, J=5.2 Hz, 2 H) 2.79 (t, J=6.0 Hz, 2 H) 6.87 - 7.00 (m, 2 H) 7.09 (s, 1 H) 7.44 - 7.56 (m, 3 H) 7.58 - 7.68 (m, 3 H) 8.22 (br. s., 2 H) 17.12 (s, 1 H). ^{13}C NMR (126 MHz, acetone) δ: 23.4, 26.7, 27.5, 107.8, 115.4, 117.2, 123.4, 127.6, 128.1, 128.3, 129.9, 130.5, 133.7, 138.2, 144.9, 146.1, 177.8, 193.8

2-benzoyl-6-(1-naphthalenylmethylene)cyclohexen-1-ol (11). Yellow amorphous powder; 68.12%; m.p.: 109-110ºC; Mass calculated: 340.1463; Mass found: 340.147. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.64 (quin, J=6.0 Hz, 2 H) 2.56 (t, J=5.8 Hz, 2 H) 2.63 (t, J=5.4 Hz, 2 H) 7.43 - 7.56 (m, 7 H) 7.59 - 7.64 (m, 2 H) 7.84 (d, J=8.2 Hz, 1 H) 7.87 - 7.91 (m, 1 H) 8.03 - 8.08 (m, 1 H) 8.31 (s, 1 H) 16.74 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.7, 27.5, 27.6, 108.4, 124.9, 125.1, 126.1, 126.4, 126.9, 127.6, 128.2, 128.5, 128.5, 130.6, 131.4, 131.9, 133.5, 133.5, 134.4, 138.5, 175.5, 196.4

2-benzoyl-6-(2-naphthalenylmethylene)cyclohexen-1-ol (12). Yellow amorphous powder; 63.68%; m.p.: 114-115ºC; Mass calculated: 340.1463; Mass found: 340.1488. ^{1}H NMR (500 MHz, $CDCl_3$) δ:1.71 (quin, J=5.8 Hz, 2 H) 2.57 (t, J=5.7 Hz, 2 H) 2.89 (t, J=5.5 Hz, 2 H) 7.42 - 7.53 (m, 5 H) 7.55 - 7.64 (m, 3 H) 7.81 - 7.89 (m, 3 H) 7.93 (br. s., 2 H) 16.80 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.2, 27.7, 108.5, 126.4, 126.7, 127.6, 127.6, 127.9, 128.1, 128.3, 129.7, 130.6, 132.8, 132.9, 133.2, 133.4, 133.9, 138.3, 176.2, 195.5

2-benzoyl-6-(2,3-dimethoxybenzylidene)cyclohexen-1-ol (13). Orange amorphous powder; 53.72%; m.p.: 69-71 ºC; Mass calculated: 350.1518; Mass found: 350.1535. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.65 (dt, J=11.9, 6.2 Hz, 2 H) 2.53 (t, J=5.8 Hz, 2 H) 2.66 (t, J=5.4 Hz, 2 H) 3.84 (s, 3 H) 3.89 (s, 3 H) 6.93 (dd, J=16.0, 7.8 Hz, 2 H) 7.04 - 7.09 (m, 1 H) 7.41 - 7.49 (m, 3 H) 7.56 - 7.59 (m, 2 H) 7.89 (s, 1 H) 16.69 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.3, 27.6, 55.9, 61.1, 108.2, 112.4, 122.1, 123.4, 127.5, 128.1,

ACCEPTED MANUSCRIPT

128.9, 130.5, 130.7, 133.5, 138.4, 148.0, 152.8, 175.9, 195.9

2-benzoyl-6-(2,5-dimethoxybenzylidene)cyclohexen-1-ol (14). Yellow amorphous powder; 43.89%; m.p.: 128-129ºC; Mass calculated: 350.1518; Mass found: 350.1545. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.66 (quin, J=6.0 Hz, 2 H) 2.53 (t, J=5.8 Hz, 2 H) 2.71 (t, J=5.4 Hz, 2 H) 3.80 (s, 3 H) 3.84 (s, 3 H) 6.85 (d, J=1.5 Hz, 2 H) 6.91 (s, 1 H) 7.40 - 7.50 (m, 3 H) 7.55 - 7.59 (m, 2 H) 7.91 (s, 1 H) 16.71 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.3, 27.6, 55.8, 56.1, 108.2, 111.5, 114.0, 116.4, 126.1, 127.6, 128.1, 128.9, 130.5, 132.7, 138.5, 152.4, 152.9, 176.1, 195.6

2-benzoyl-6-(3,4-dimethoxybenzylidene)cyclohexen-1-ol (15). Yellow amorphous powder; 42.21%; m.p.: 152-153ºC; Mass calculated: 350.1518; Mass found: 350.1542 ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (quin, J=6.0 Hz, 2 H) 2.52 - 2.57 (m, 2 H) 2.76 - 2.84 (m, 2 H) 3.92 (s, 3 H) 3.92 (s, 3 H) 6.91 (d, J=8.5 Hz, 1 H) 7.00 (d, J=1.8 Hz, 1 H) 7.09 (dd, J=8.3, 1.6 Hz, 1 H) 7.41 - 7.49 (m, 3 H) 7.58 (dd, J=7.7, 1.6 Hz, 2 H) 7.72 (s, 1 H) 16.89 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.1, 27.7, 55.9, 56.0, 108.1, 110.9, 113.5, 123.5, 127.7, 128.1, 129.3, 130.5, 130.8, 133.5, 138.3, 148.7, 149.3, 177.2, 194.4

2-benzoyl-6-(3,4,5-trimethoxybenzylidene)cyclohexen-1-ol (16). Yellow amorphous powder; 27.85%; m.p.: 150-151ºC; Mass calculated: 380.1624; Mass found: 380.1631. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (t, J=5.7 Hz, 2 H) 2.54 (t, J=6.0 Hz, 2 H) 2.80 (t, J=5.2 Hz, 2 H) 3.89 (s, 6 H) 3.89 (s, 3 H) 6.69 (s, 2 H) 7.42 - 7.48 (m, 3 H) 7.58 (dd, J=7.9, 1.5 Hz, 2 H) 7.69 (s, 1 H) 16.79 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.6, 27.1, 27.7, 56.2, 56.2, 61.0, 107.6, 108.3, 127.6, 128.1, 130.6, 131.8, 131.9, 131.9, 133.5, 138.2, 138.4, 153.0, 176.4, 195.2

2-benzoyl-6-(4-hydroxyl-3-methoxybenzylidene)cyclohexen-1-ol (17). Yellow; 51.66%; m.p.: 153-154ºC; Mass calculated: 336.1362; Mass found: 336.13822. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.67 (quin, J=6.0 Hz, 2 H) 2.53 (t, J=5.8 Hz, 2 H) 2.78 (t, J=5.4 Hz, 2 H) 3.93 (s, 3 H) 5.67 (s, 1 H) 6.89 (d, J=8.4 Hz, 1 H) 7.00 (d, J=8.5 Hz, 1 H) 7.10 (s, 1 H) 7.39 - 7.49 (m, 3 H) 7.58 (d, J=6.7 Hz, 2 H) 7.68 (s, 1 H) 16.88 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.5, 27.1, 27.7, 56.0, 108.1, 110.4, 115.9, 123.4, 127.7, 128.1, 129.8, 130.5, 131.0, 133.4, 138.3, 145.3, 146.8, 177.3, 194.4

2-benzoyl-6-(3-chloro-4-hydroxybenzylidene)cyclohexen-1-ol (18). Yellow amorphous powder; 62.13%; m.p.: 164-165ºC; Mass calculated: 340.0866; Mass found: 340.0878. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (quin, J=6.0 Hz, 2 H) 2.51 - 2.56 (m, 2 H) 2.69 - 2.78 (m, 2 H) 5.74 (s, 1 H) 7.05 (d, J=8.4 Hz, 1 H) 7.30 (dd, J=8.6, 1.9 Hz, 1 H) 7.42 - 7.50 (m, 4 H) 7.56 - 7.60 (m, 2 H) 7.63 (s, 1 H) 16.75 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.5, 27.1, 27.6, 108.4, 116.2, 120.0, 127.6, 128.1, 130.1, 130.5, 130.6, 130.7, 131.7, 131.8, 138.2, 151.3, 176.3, 195.2

2-benzoyl-6-(3-bromo-4-hydroxybenzylidene)cyclohexen-1-ol (19). Yellow amorphous powder; 65.19%; m.p.: 172-173ºC; Mass calculated: 384.0361; Mass found: 384.0379. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (quin, J=6.0 Hz, 2 H) 2.50 - 2.57 (m, 2 H) 2.71 - 2.78 (m, 2 H) 5.70 (br. s., 1 H) 7.05 (d, J=8.5 Hz, 1 H) 7.34 (dd, J=8.5, 2.0 Hz, 1 H) 7.41 - 7.50 (m, 3 H) 7.55 - 7.61 (m, 3 H) 7.63 (s, 1 H) 16.74 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.5, 27.1, 27.6, 108.3, 110.3, 116.0, 127.6, 128.1, 130.5, 130.6, 131.4, 131.5, 131.9, 133.6, 138.2, 152.2, 176.2, 195.2

2-benzoyl-6-(5-methyl-2-furanylmethylene)cyclohexen-1-ol (20). Red amorphous powder; 44.94%; m.p.: 104-106ºC; Mass calculated: 294.1256; Mass found: 294.1265. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.72 (quin, J=6.1 Hz, 2 H) 2.38 (s, 3 H) 2.50 - 2.55 (m, 2 H) 2.84 (t, J=5.5 Hz, 2 H) 6.13 (d, J=2.3 Hz, 1 H) 6.54 (d, J=3.2 Hz, 1 H) 7.40 - 7.48 (m, 4 H) 7.57 (dd, J=7.7, 1.6 Hz, 2 H) 16.85 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.0, 23.0, 27.0, 27.4, 108.2, 108.9, 116.6, 120.5, 127.7, 127.9, 128.1, 130.4, 138.3, 151.6, 154.8, 177.4, 193.6

2-benzoyl-6-(thien-2-ylmethylene)cyclohexen-1-ol (21). Orange amorphous powder; 54.28%; m.p.: 110-111ºC; Mass calculated: 296.0871; Mass found: 296.0896. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.77 (dt, J=12.1, 6.2 Hz, 2 H) 2.52 - 2.58 (m, 2 H) 2.75 - 2.82 (m, 2 H) 7.14 (dd, J=5.0, 3.8 Hz, 1 H) 7.33 (d, J=3.5 Hz, 1 H) 7.43 - 7.48 (m, 3 H) 7.51 (d, J=5.0 Hz, 1 H) 7.58 (dd, J=7.9, 1.5 Hz, 2 H) 7.95 (s, 1 H) 16.87 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.0, 26.8, 27.7, 108.4, 126.5, 127.6, 127.7, 128.1, 129.3, 129.4, 130.5, 131.9, 138.1, 139.9, 177.3, 193.8

2-benzoyl-6-(5-methyl-2-thienylmethylene)cyclohexen-1-ol (22). Yellow amorphous powder; 58.16%; m.p.: 100-102ºC; Mass calculated: 310.1028; Mass found: 310.1035. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.75 (quin, J=6.2 Hz, 2 H) 2.49 - 2.58 (m, 5 H) 2.75 (t, J=5.4 Hz, 2 H) 6.79 - 6.82 (m, 1 H) 7.14 (d, J=3.5 Hz, 1 H) 7.39 - 7.48 (m, 3 H) 7.57 (dd, J=7.9, 1.5 Hz, 2 H) 7.86 (s, 1 H) 16.96 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 15.7, 23.0, 26.8, 27.6, 108.1, 126.1, 127.2, 127.8, 128.0, 128.1, 130.4, 132.5, 138.0, 138.1, 144.9, 178.0, 193.0

2-benzoyl-6-(3-fluorobenzylidene)cyclohexen-1-ol (23). Yellow; 68.57%; m.p.: 121-122ºC; Mass calculated: 308.1213; Mass found: 308.1228. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.68 (quin, J=6.1 Hz, 2 H) 2.51 - 2.58 (m, 2 H) 2.73 - 2.79 (m, 2 H) 7.02 (td, J=8.5, 1.8 Hz, 1 H) 7.14 (d, J=10.2 Hz, 1 H) 7.21 (d, J=7.6 Hz, 1 H) 7.36 (td, J=8.0, 6.1 Hz, 1 H) 7.41 - 7.51 (m, 3 H) 7.55 - 7.61 (m, 2 H) 7.70 (s, 1 H) 16.64 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$ $CDCl_3$) δ: 23.5, 27.2, 27.5, 108.7, 115.0, 116.5, 125.9, 127.6, 128.2, 129.9, 130.7, 131.8, 133.6, 138.2, 138.5, 163.6, 175.3, 196.1

2-benzoyl-6-(2,3,4,-trimethoxybenzylidene)cyclohexen-1-ol (24). Yellow amorphous powder; 41.21%; m.p.: 140-141ºC; Mass calculated: 380.1624; Mass found: 380.1643. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.63 - 1.69 (m, 2 H) 2.53 (t, J=5.8 Hz, 2 H) 2.69 (t, J=5.2 Hz, 2 H) 3.90 (s, 6 H) 3.91 (s, 3 H) 6.69 (d, J=8.7 Hz, 1 H) 7.09 (d, J=8.7 Hz, 1 H) 7.40 - 7.48 (m, 3 H) 7.55 - 7.59 (m, 2 H) 7.88 (s, 1 H) 16.80 (s, 1 H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 23.7, 27.3, 27.7, 56.1, 61.0, 61.5, 106.7, 107.9, 123.3, 125.0, 127.6, 128.1, 128.7, 130.4, 131.8, 138.5, 142.3, 153.1, 154.1, 176.8, 195.1

29. **Cell-based NO Inhibitory Assay. Cell culture.** ATCC RAW264.7 murine macrophages were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin in a 95% air and 5% CO_2 atmosphere at 37 °C. **Nitrite determination.** RAW 264.7 cells at 90-95% confluency were detached and seeded (50000 cells/well) into a 96-well culture plate with 50 µL of DMEM and incubated for 24 h. The cells were then stimulated in 5 mg/mL of LPS (Escherichia coli, serotype 0111:B4) and 1 ng/ml of interferon-gamma (IFN-γ) in the presence or absence of test compounds for 17 hours. Nitrite concentration was then determined by Griess assay by reacting 50 µL of cell culture supernatant with 50 µL of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5%

ACCEPTED MANUSCRIPT

phosphoric acid) at room temperature. The optical density was measured at 550 nm after 5 minutes of incubation at room temperature with a microplate reader. **Cell cytotoxicity determination (MTT assay).** Supernatant in each well was removed followed by addition of 100 µL DMEM. Following this, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was then added and the plate was incubated in a 95% air and 5% CO_2 atmosphere at 37 °C for 4 hours. The mixture of culture media and MTT in all wells were removed and the purplish formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) and further incubated for 15 minutess at room temperature. Color intensity was then measured at 570 nm at room temperature.

30. Bogdan, C. *Nat. Immunol.* **2001**, *2*, 907.
31. Ridnour, L. A.; Thomas, D. D.; Switzer, C.; Flores-Santana, W.; Isenberg, J. S.; Ambs, S.; Roberts, D. D.; Wink, D. A. *Nitric Oxide* **2008**, *19*, 73.
32. Naseem, K. M. *Mol. Aspects Med.* **2005**, *26*, 33.
33. Hensley, K.; Tabatabaie, T.; Stewart, C. A.; Pye, Q.; Floyd, R. A. *Chem. Res. Toxicol.* **1997**, *10*, 527.
34. Sellebjerg, F.; Giovannoni, G.; Hand, A.; Madsen, H. O.; Jensen, C. V.; Garred, P. *J. Neuroimmunol.* **2002**, *125*, 198.
35. Marnett, L. J. *J. Org. Chem.* **2012**, *77*, 5224.
36. Lam, K. W.; Tham, C. L.; Liew, C. Y.; Syahida, A.; Rahman, M. B. A.; Israf, D. A.; Lajis, N. H. *Med. Chem. Res.* **2012**, *21* , 333.
37. Chuprajob, T.; Changtam, C.; Chokchaisiri, R.; Chunglok, W.; Sornkaew, N.; Suksamrarn, A. *Bioorg. Med. Chem. lett.* **2014**, *24*, 2839.
38. Shin, S. Y.; Shin, M. C.; Shin, J. S.; Lee, K. T.; Lee, Y. S. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4520.
39. Simpsonb, C. J.; Fitzhenrya, M. J.; Stamford, N. P. J. *Tetrahedron Letters* **2005**, *46*, 6893.
40. Sivakumar, P. M.; Geetha Babu, S. K.; Mukesh, D. *Chem. Pharm. Bull*. **2007**, *55*, 44.
41. Chiang, Y. M.; Lo, C. P.; Chen, Y. P.; Wang, S. Y.; Yang, N. S.; Kuo, Y. H.; Shyur, L. F. *Br. J. Pharmacol.* **2005**, *146*, 352.
42. **Antioxidant evaluations. DPPH Free Radical Scavenging Assay.** Generally 100 µL of DPPH solution (100 µM) and 100 µL of sample dissolved in DMSO (100 µM) were added to a 96-well microplate. The mixture was then shaken gently and incubated in the dark for 30 minutes followed by absorbance measurement at 517 nm. Active compounds were further analyzed through serial dilution technique to obtain their IC_{50} value. **FRAP Assay.** Generally 20 µL of sample or standard dissolved in 3% DMSO in buffer was added to a 96-well microplate in triplicate, followed by the addition of potassium phosphate buffer (40 µL, 0.2 M, pH 7.2) and potassium ferricyanide (40 µL, 1% w/v). The reaction mixtures were incubated at 50 ◦C for 20 min followed by adding 40 µL of 10% w/v trichloroacetic acid, 150 µL of distilled water and 30 µL of 0.1% w/v $FeCl_3$. The reaction mixtures were further incubated at room temperature for 30 min in the dark. Absorbance readings were measured at 630 nm. Active compounds were further analyzed through serial dilution technique to obtain a dose-dependent graph. **Nitrite Scavenging Assay.** Briefly, 75 µL of SNP (10 mM) solution were incubated alone or in combination with 75 µL of 500 µM test compounds for 60 min with light exposure at room temperature. The nitrite levels of the mixture were then determined by adding Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid). The absorbance was measure at 550 nm.
43. Colclough, N.; Hunter, A.; Kenny, P. W.; Kittlety, R. S.; Lobedan, L.; Tam, K. Y.; Timms, M. A. *Bioorg. Med. Chem.* **2008**, *16*, 6611.
Solubility Test. A DMSO stock solution of 10 mM curcumin or active compound **10** were prepared and diluted with phosphate buffer (pH 7.4) in the ratio of 1 to 99. The respective mixtures were stirred for 24 hours at room temperature to achieve equilibrium. The mixtures were then filtered through a 0.45 µm syringe filter. The filtrates were further diluted with HPLC grade methanol as co-solvent in the ratio of 1 to 2 and the content was measured by the quantitative reverse phase HPLC analysis on a 4.3 x 150 mm XBridge™ C18 column (5 µm, Part no: 186003116). The mobile phase was composed of 0.1% formic acid and methanol for both curcumin (40:60) and compound **10** (30:70). A flow rate of 1.0 ml/min was used. Curcumin and compound **10** were detected at 360 nm.
44. USP 38 NF 33. The United States Pharmacopeia Convention, Rockville, MD
45. Zhang, Y.; Zhao, C.; He, W.; Wang, Z.; Fang, Q.; Xiao, B.; Liu, Z.; Liang, G.; Yang, S., *Drug. Des. Devel. Ther.* **2014**, *8*, 373.
Chemical Stability Test. A stock solution of 50 mM curcumin or active compound **10** were prepared and diluted by phosphate buffer (pH 7.4), containing 1% dimethyl sulfoxide (DMSO), to a final concentration of 20 µM. The ultraviolet absorption spectra were collected for over 30 min at 5-min intervals at 25 °C. All spectral measurements were carried out in a 1 cm path-length quartz cuvette. Absorbance readings were taken from 300–600 nm.
46. Stout, E. P.; Choi, M. Y.; Castro, J. E.; Molinski, T. F. *J. Med. Chem.* **2014**, *57*, 5085.
Microsomal Stability Test. A mixture of pH 7.4 phosphate buffer (100 mM, 183 µL), human liver microsome solution (20 mg/mL, 5 µL) and test sample (1 mM, 2 µL) were pre-incubated at 37 °C for 5 minutes. The reaction was commenced upon the addition of NAPDH solution (40mM, 10 µL) and further incubated at 37 °C for different time points (0, 15, 30, 45, 60, 90 and 120 minutes). The reaction was quenched by the addition of 200 µL HPLC grade methanol followed by vortex homogenization and centrifugation for 5 minutes. The concentration of the remaining test sample in supernatant was determined by quantitative HPLC analysis as described above.