Bioorganic & Medicinal Chemistry Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of new heterocyclic lupeol derivatives as nitric oxide and pro-inflammatory cytokine inhibitors

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ARTICLE INFO

Article history:
Received 5 March 2014
Revised 9 May 2014
Accepted 12 May 2014
Available online xxxx

Keywords:
Lupeol
Heterocyclic derivatives
Anti-inflammatory activity
Nitric oxide
Tumor necrosis factor-alpha

ABSTRACT

A series of heterocyclic derivatives including indoles, pyrazines along with oximes and esters were synthesized from lupeol and evaluated for anti-inflammatory activity through inhibition of lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 and J774A.1 cells. All the synthesized molecules of lupeol were found to be more active in inhibiting NO production with an IC_{50} of 18.4–48.7 μM in both the cell lines when compared to the specific nitric oxide synthase (NOS) inhibitor, L-NAME (IC_{50} = 69.21 and 73.18 μM on RAW 264.7 and J774A.1 cells, respectively). The halogen substitution at phenyl ring of indole moiety leads to potent inhibition of NO production with half maximal concentration ranging from 18.4 to 41.7 μM. Furthermore, alkyl (**11**, **12**) and *p*-bromo/iodo (**15**, **16**) substituted compounds at a concentration of 20 μg/mL exhibited mild inhibition (29–42%) of LPS-induced tumor necrosis factor alpha (TNF-α) and weak inhibition (10–22%) towards interleukin 1-beta (IL-1β) production in both the cell lines. All the derivatives were found to be non-cytotoxic when tested at their IC_{50} (μM). These findings suggest that the derivatives of lupeol could be a lead to potent inhibitors of NO.

Injury leads to inflammation which involves the assemblage of cells and exudates in irritated tissues.[1] Chronic inflammatory diseases such as psoriasis, ulcerative colitis and rheumatoid arthritis usually occurred due to ungoverned production of pro-inflammatory cytokines.[2] Among the cytokines, nitric oxide (NO) is a potent pro-inflammatory mediator and at low concentration regulates physiological homeostasis in the cardiovascular system.[3] Overproduction of NO by inducible nitric oxide synthase (*i*NOS) generally destroys functional normal tissues during acute and chronic inflammation.[4] Tumor necrosis factor-alpha (TNF-α), another pro-inflammatory cytokine plays an important role in inflammation cascades, and induces itself as well as other inflammatory cytokines.[5]

Natural products represent promising scaffolds with high chemical and structural diversity and wide array of biological activities. Out of the structurally diverse compounds from tropical plants, the triterpenoids are often found in significant quantities, and a wide array isolated and characterized. Lup-20(29)-en-3β-ol, commonly known as lupeol, a pentacyclic triterpenoid with 30 carbon atoms, biosynthetically derived from the cyclization of squalene has a vast occurrence in diverse plant families. Since the triterpenes are widely distributed in food, medicinal herbs and other plants, these compounds can be an integral part of the human diet.[6] Consequently, much research interest has been focused on the pharmacological evaluation and efficient isolation of triterpenoids from plants.[7,8] Lupeol had been studied for more than a century and is reported to exhibit a broad spectrum of pharmacological activities against some prominent disease such as inflammation, diabetes, arthritis, cardiovascular ailments, renal disorders, hepatic toxicity, microbial infections and cancer.[9–20] Earlier studies on the synthetic modifications of lupeol resulted in the preparation of antimalarial, antidiabetic and antihypoglycemaic analogues.[21,22] Accordingly, chemical modifications, of the structure of lupeol, were required to improve the biological activities while showing less toxicity. Therefore, in order to find potent anti-inflammatory derivatives, in present work, considerable structural modifications have been performed mainly on the A ring. The Fischer indolization of lupeol, modifications of ring A at C-3 position and the isoprenyl unit at C-30 resulted in the preparation of different derivatives which were evaluated for anti-inflammatory activity on RAW 264.7 and J774A.1 cells along with their cytotoxicity.

Lupeol was isolated from the bark of *Embelica officinalis* in good yield (1.4%). For the isolation of lupeol, the air dried bark of *E. officinalis* was ground to fine powder and extracted with *n*-hexane and the combined percolations were concentrated to dryness under reduced pressure. The lupeol was isolated by

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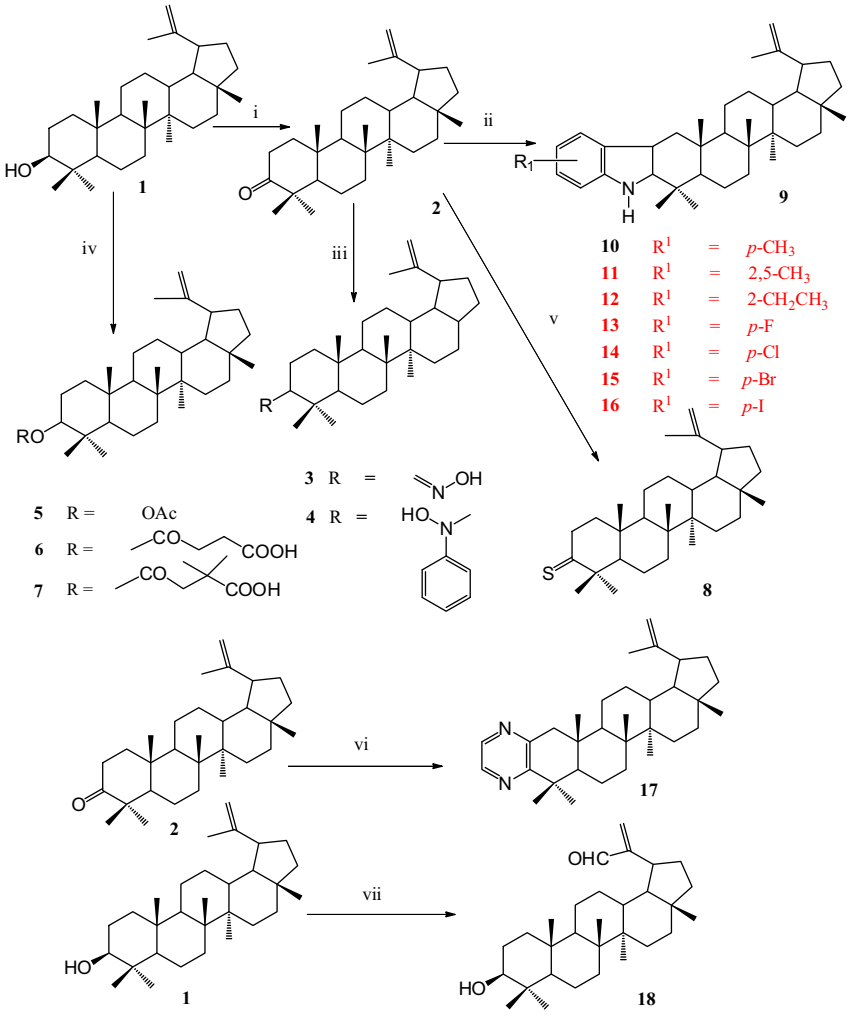
http://dx.doi.org/10.1016/j.bmcl.2014.05.032
0960-894X/

column chromatography on silica gel with 5% EtOAc/*n*-hexane and re-crystallization.

The synthesized derivatives are divided into six categories: 3-keto lupeol **2**; 3-oxime **3** and **4**; 3-ester derivatives **5**–**7**; sulfur derivative **8**; indole derivatives **9**–**16**; pyrazine derivatives **17**; and allylic oxidation (an aldehyde) **18**. Scheme 1 describes the synthesis of derivatives **2**–**18**. 3-Keto lupeol (**2**), a key intermediate, was prepared by Jone's oxidation of **1** in 91% yields. Compounds **3** and **4** were prepared by refluxing **2** with hydroxylamines in the presence of pyridine (anhydrous) under a nitrogen atmosphere. The synthesis of **5**–**7** was accomplished by esterification of **1** with anhydrides in presence of DMAP/dry pyridine (Scheme 1). Compound **8** was prepared by thionation of **2** using Lawesson's reagent in toluene. The indole derivative (**9**) of lupeol was prepared by the Fischer indolization of **2** with phenylhydrazine hydrochloride in the presence of acetic acid. ^{1}H and ^{13}C NMR data of **9** suggested the disappearance of H-2 proton and keto group at C-3 (δ 217.2) of 3-oxolupeol (**2**). The NMR spectrum data of **9** was closely similar with lupeol with additional signals at δ_H 7.72 (NH), and aromatic proton signals at δ_H 7.03–7.39. In order to understand the influence on the activity of a substitution group at phenyl ring, compounds **10**–**16** were prepared by the same protocol as **9**. It is noteworthy that the electron donating groups substituted to the phenyl ring enhances the anti-inflammatory activity. The reaction of ketone **2** with ethylenediamine and sulfur in refluxing morpholine followed by silica gel chromatography has been applied to provide pyrazine derivative. To introduce aldehyde group on the allylic position of lupeol, allylic oxidation with SeO_2 under reflux in moist dioxane followed by silica gel chromatography was carried out to afford compound **18**. In NMR spectrum, a new proton at δ_H 9.86 appeared along with olefinic protons.

Nitric oxide (NO) is an important mediator in the inflammatory process and generated normally at the inflammatory sites by an inducible nitric oxide synthase (NOS) enzyme. Macrophages alone itself released low levels of NO and upon stimulation with gram negative bacteria derived components such as lipopolysaccharide (LPS), the production of NO raised appreciably up to 24 h. The NO inhibitory effects of lupeol (**1**), and its derivatives (3-keto **2**, oximes **3**,**4**, esters **5**–**7** heterocyclic derivatives **9**–**17**, and aldehyde **18**) on RAW 264.7 and J774A.1 cells was judged by using Griess reaction[23] while cell viability was estimated using conventional MTT assay[24] (Table 1). Results revealed that all the derivatives of lupeol were found to be more active in inhibiting NO production with an IC_{50} of 18.4 to 48.7 μM in both the cell lines when compared to the specific inhibitor, L-NAME (IC_{50} = 69.21 and 73.18 μM on RAW 264.7 and J774A.1 cells, respectively). However, in comparison to curcumin (IC_{50} = 11.02 and 18.52 μM on RAW 264.7 and J774A.1 cells, respectively), only compound **15** showed significant activity (IC_{50} = 23.9 and 18.4 μM on RAW 264.7 and J774A.1 cells, respectively) towards NO inhibition. Compounds



Scheme 1. (i) Jone's oxidation (ii) phenylhydrazine derivatives, AcOH, reflux, (iii) Hydroxylamine hydrochloride, pyridine (anhydrous), reflux, (iv) Anhydrides, DMAP, pyridine (anhydrous), reflux, (v) Lawesson's reagent, toluene, (vi) ethylenediamine, morpholine, sulfur, reflux, (vii) SeO_2, dioxane (moist), reflux.

Please cite this article in press as: Bhandari, P.; et al. *Bioorg. Med. Chem. Lett.* (2014), http://dx.doi.org/10.1016/j.bmcl.2014.05.032

Table 1
NO inhibitory effects of compounds on RAW 264.7 and J774A.1 cells

Compounds	IC_{50}(μM) for inhibition of NO production		Cell viability[a] (% of control)	
	RAW 264.7	J774A.1	RAW 264.7	J774A.1
[b]L-NAME	69.21 ± 2.65	73.18 ± 1.70	—	—
[b]Curcumin	11.02 ± 2.40	18.52 ± 2.89	—	—
1	37.3 ± 0.34	34.5 ± 2.50	87.30 ± 1.89	88.27 ± 1.63
2	38.5 ± 0.37	38.8 ± 4.20	89.60 ± 3.29	87.72 ± 1.90
3	46.0 ± 0.28	44.6 ± 1.40	95.72 ± 2.50	93.64 ± 3.78
4	48.7 ± 1.38	47.7 ± 0.88	79.30 ± 1.43	82.47 ± 2.42
5	36.7 ± 1.67	33.2 ± 1.28	80.35 ± 3.82	83.51 ± 1.98
6	39.2 ± 0.78	39.3 ± 0.97	91.29 ± 0.67	90.13 ± 2.82
7	39.2 ± 1.48	42.6 ± 1.36	87.56 ± 2.06	83.64 ± 1.75
8	36.2 ± 2.67	36.3 ± 1.48	94.29 ± 0.04	91.93 ± 2.72
9	31.7 ± 1.54	31.7 ± 1.27	91.83 ± 4.10	90.34 ± 1.19
10	27.8 ± 2.93	28.6 ± 2.89	89.30 ± 3.18	87.02 ± 2.39
11	25.9 ± 3.17	23.2 ± 0.98	93.24 ± 1.29	91.46 ± 3.20
12	30.6 ± 2.76	28.6 ± 2.56	91.47 ± 2.84	91.74 ± 1.95
13	41.7 ± 1.38	41.7 ± 2.60	95.20 ± 1.76	92.08 ± 2.07
14	35.6 ± 2.45	33.2 ± 4.10	93.27 ± 2.85	92.74 ± 1.19
15	23.9 ± 1.34	18.4 ± 3.04	91.38 ± 0.94	90.85 ± 3.76
16	22.6 ± 2.75	25.2 ± 2.89	95.23 ± 1.05	93.34 ± 2.03
17	32.4 ± 1.38	32.4 ± 2.30	95.23 ± 1.05	93.34 ± 2.03
18	42.9 ± 2.45	37.0 ± 1.27	95.20 ± 1.76	92.08 ± 2.07

Values are represented as mean ± SD of three different experiments in triplicates.
[a] Cell viability was measured at IC_{50} (μM).
[b] Standards used for the present study.

10–**12**, **15** and **16** were observed to be more potent than other synthesized derivatives and parent compound. Compounds **15** and **16**, (substitution of halogens in the phenyl ring of indole moiety) showed most potent inhibition (IC_{50} = 23. 9 and 22.6 μM in RAW 264.7 cells and IC_{50} = 18. 4 and 25.2 μM in J774A.1 cells, respectively) of NO production followed by compounds **11** and **12** (IC_{50} = 25. 9 μM and 30.6 μM in RAW 264.7 cells and IC_{50} = 23.2 and 28.6 μM in J774A.1 cells, respectively). Furthermore, in comparison to the parent compound lupeol (**1**), activity reduces with **2** and **5**–**7** with a half maximal concentration 38.5 to 39.2 μM while no apparent change in NO inhibition (IC_{50} ~41.7–48.7 μM) was observed with **3**, **4**, **13** and **18**. Also, compounds **8**, **9**, **14** and **17** depicted moderate to mild inhibition of NO with IC_{50} ranging from 27.8 to 36.2 μM. The results revealed that indole moieties containing only one nitrogen atom had a noticeable impact on the activity. However, substitution of the halogen group (bromine and iodine) on the phenyl ring of indole moiety resulted in the significant inhibition. It is important to note that substitution of electron donating group at the phenyl ring of indole moiety that is ethyl, methyl and dimethyl also exhibit potent inhibition effects.

Similar to NO, macrophages also released basal levels of inflammatory cytokines and upon stimulation with LPS, the production of these mediators increased markedly starting from 4 h and lasts mostly up to 18 h. In view of significant NO inhibitory effects, compounds **11**, **12**, **15** and **16** at a concentration of 20 μg/mL were further tested for the reduction of LPS-stimulated TNF-α and IL-1β release of RAW 264.7 and J774A.1 cells using the protocol provided in the enzyme linked immune-sorbent assay (ELISA) kit.[24] The results revealed that these compounds exhibited mild inhibition (29–42%) of LPS-induced tumor necrosis factor alpha (TNF-α) whereas found to be inactive (10–22%) towards IL-1β inhibition (Table 2).

Taking the above results together, a preliminary structure activity relationship (SAR) for lupeol derivatives could be outlined as follows: (1) A carbonyl group at C-3 of lupeol could enhance the activity. (2) Substituted groups on the phenyl ring of indole moiety could promote the activity. (3) A hydrophobic group (methyl ester) at the C-3 of the lupeol could reduce the activity.

Table 2
TNF-α and IL-1β inhibitory effects of compounds on RAW 264.7 and J774A.1 cells at concentration of 20 μg/mL

Compounds	TNF-α (% inhibition)		IL-1β (% inhibition)	
	RAW 264.7	J774A.1	RAW 264.7	J774A.1
[a]Curcumin	1.25 ± 2.15	2.08 ± 3.56	6.44 ± 1.55	24.82 ± 2.93
[a]Dexamethasone	0.016 ± 1.67	0.165 ± 1.29	1.84 ± 2.35	9.09 ± 3.51
11	32.8 ± 1.32	31.1 ± 2.34	22.8 ± 1.29	17.2 ± 3.29
12	39.1 ± 4.20	29.4 ± 0.39	11.4 ± 3.17	10.1 ± 1.36
15	39.2 ± 2.94	31.5 ± 3.49	19.7 ± 1.27	16.2 ± 1.25
16	42.3 ± 4.38	37.0 ± 1.28	22.9 ± 3.29	17.3 ± 3.25

Values are represented as mean ± SD of three different experiments in triplicates.
[a] Standards used for the present study (IC_{50} in μg/mL).

Therefore taking the above results together, a conclusive study showed that substitution of the alkyl and halogen groups on the phenyl ring of indole moiety enhances the inhibition towards NO production. Compounds **10**–**12**, **15** and **16** exhibited potent anti-inflammatory activity mainly through inhibition of NO release from RAW 264.7 and J774A.1 cells. It seems that presence of six membered heterocyclic ring with two nitrogen atoms at C-2 and C-3 of lupeol did not impact on the activity. However, substituted electron donating groups at the phenyl ring of indole moiety promotes the activity. The alkyl (**11**, **12**) and halogen substituted indole (**15**, **16**) also found to be moderately active towards TNF-α release while none of the synthesized derivatives was active towards IL-1β on RAW 264.7 and J774A.1 cells. The aforementioned results suggested that derivatives of lupeol could be a lead to potent inhibitors of NO.

Acknowledgements

The authors gratefully acknowledge the financial support from the Director, NIPER, SAS Nagar, India for the research work. N.K.P. is a recipient of DST-INSPIRE Senior Research Fellowship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.032.

References and notes

1. Vane, J.; Botting, R. *FASEB J.* **1987**, *1*, 89.
2. Palladino, M. A.; Bahjat, F. R.; Theodorakis, E. A.; Moldawer, L. L. *Nat. Rev. Drug Discov.* **2003**, *2*, 736.
3. Hesslinger, C.; Strub, A.; Boer, R.; Ulrich, W. R.; Lehner, M. D.; Braun, C. *Biochem. Soc. Trans.* **2009**, *37*, 886.
4. Knowles, R. G.; Moncada, S. *J. Biochem.* **1994**, *298*, 249.
5. Yamazaki, Y.; Kawano, Y. *Chem. Pharm. Bull.* **2011**, 388.
6. Tian, Z.; Lin, G.; Zheng, R. X.; Huang, F.; Yang, M. S.; Xiao, P. G. *World J. Gastroenterol.* **2006**, *12*, 874.
7. Kowalski, R. *J. Agric. Food Chem.* **2007**, *55*, 656.
8. Liu, J. *J. Ethnopharmacol.* **1995**, *49*, 57.
9. Moriarity, D. M.; Huang, J.; Yancey, C. A.; Zhang, P.; Setzer, W. N.; Lawton, R. O.; Bates, R. B.; Caldera, S. *Planta Med.* **1998**, *64*, 370.
10. Al-Rehaily, A. J.; El-Tahir, K. E. H.; Mossa, J. S.; Rafatullah, S. *Nat. Prod. Sci.* **2001**, *7*, 76.
11. Chaturvedi, P. K.; Bhui, K.; Shukla, Y. *Cancer Lett.* **2008**, *263*, 1.
12. Fernandez, M. A.; de las Heras, B.; García, M. D.; Saenz, M. T.; Villar, A. *J. Pharm. Pharmcol.* **2001**, *53*, 1533.
13. Fernandez, A.; Alvarez, A.; Garcia, M. D.; Saenz, M. T. *Farmacologiche* **2001**, *56*, 335.
14. Fulda, S.; Debatin, K. M. *Med. Pediatr. Oncol.* **2000**, *35*, 616.
15. Geetha, T.; Varalakshmi, P. *J. Ethnopharmacol.* **2001**, *76*, 77.
16. Lima, L. M.; Perazzo, F. F.; Tavares Carvalho, J. C.; Bastos, J. K. *J. Pharm.* **2007**, *59*, 1151.
17. Recio, M. C.; Giner, R. M.; Manez, S.; Gueho, J.; Julien, H. R.; Hostettmann, K.; Ríos, J. L. *Planta Med.* **1995**, *61*, 9.
18. Ryu, S. Y.; Oak, M. H.; Yoon, S. K.; Cho, D. I.; Yoo, G. S.; Kim, T. S.; Kim, K. M. *Planta Med.* **2000**, *66*, 358.
19. Sudhahar, V.; Ashok Kumar, S.; Varalakshmi, P.; Sujatha, V. *Mol. Cell. Biochem.* **2008**, *317*, 11.
20. Sudhahar, V.; Veena, C. K.; Varalakshmi, P. *J. Nat. Prod.* **2008**, *71*, 1509.
21. PapiReddy, K.; Singh, A. B.; Puri, A.; Srivastava, A. K.; Narender, T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4463.
22. Srinivasan, T.; Srivastava, G. K.; Pathak, A.; Batra, S.; Raj, K.; Singh, K.; Puri, S. K.; Kundu, B. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2803.
23. Inoue, T.; Hamada, Y.; Takeshita, K.; Fukushima, K.; Higaki, M. *J. Rheumatol.* **2001**, *28*, 1229.
24. An, S. L.; Pae, H. O.; Oh, G. S.; Choi, B. M.; Jeong, S.; Jang, S. I.; Oh, H.; Known, T. O.; Song, C. E.; Chung, H. T. *Int. Immunopharmacol.* **2002**, *2*, 1173.