DOI: 10.1002/cbdv.201800269

FULL PAPER

CHEMISTRY & BIODIVERSITY

Inhibition of Pro-Inflammatory Functions of Human Neutrophils by Constituents of *Melodorum fruticosum* Leaves

Nora S. Engels,[a] Birgit Waltenberger,*[a] Barbara Michalak,[b] Loi Huynh,[c] Hung Tran,[c] Anna K. Kiss,[b] and Hermann Stuppner[a]

[a] Institute of Pharmacy/Pharmacognosy and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innrain 80–82, 6020 Innsbruck, Austria, e-mail: birgit.waltenberger@uibk.ac.at

[b] Department of Pharmacognosy and Molecular Basis of Phytotherapy, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

[c] Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy, 41–43 Đinh Tiên Hoàng, Bến Nghé, Quận 1, Hồ Chí Minh, Vietnam

In an initial screening, the dichloromethane extract from the leaves of *Melodorum fruticosum* showed distinct inhibitory effects on the release of interleukin-8 (IL-8) in human neutrophils. Therefore, the aim of the present study was the phytochemical and pharmacological investigation of this extract, to better understand which compounds might be responsible for the anti-inflammatory effect. Phytochemical analysis led to the isolation of 12 known compounds and two new natural products, 5-hydroxy-6-(2-hydroxybenzyl)-4′,7-dimethoxyflavanone (**13**) and 2′,4′-dihydroxy-3′-(2-hydroxybenzyl)-4,6′-dimethoxychalcone (**14**). The influence of the isolated compounds on the production and release of the pro-inflammatory factors IL-8, tumor necrosis factor alpha (TNF-α), reactive oxygen species (ROS), and adhesion molecules (CD62L and CD11b) in human neutrophils was evaluated. Three constituents, melodamide A, 2′,4′-dihydroxy-4,6′-dimethoxychalcone, and 2′,6′-dihydroxy-4′-methoxychalcone, showed significant inhibition of IL-8 release (IC_{50} = 6.6, 8.6, and 11.6 μM, respectively) and TNF-α production (IC_{50} = 4.5, 13.3, and 6.2 μM, respectively).

Keywords: *Melodorum fruticosum*, natural products, inflammation, cytotoxicity, neutrophils, cytokines.

Introduction

Inflammation is a complex immune response following tissue injury or infection. As part of an innate immune response, circulating neutrophils (polymorphonuclear leukocytes) are activated. They migrate towards the site of inflammation by adhesion to endothelial cells followed by migration across vascular walls to the site of infection, where they engulf invading pathogens through phagocytosis, generate reactive oxygen metabolites, and release microbicidal, chemotactic, and pro-inflammatory substances and lytic enzymes.[1,2] Neutrophils also contribute to the resolution of inflammation through the production of pro-resolving lipid mediators, blocking chemokines and cytokines, and finally by entering into apoptosis.[3] However, inappropriate or excessive activation of neutrophils cause host tissue damage and may lead to chronic inflammation which is the basis of many chronic diseases such as atherosclerosis, bowel diseases (IBD, Crohn's disease, and ulcerative colitis), and rheumatoid arthritis.[4–6]

The current treatment of inflammatory disorders in Western medicine involves the use of non-steroidal anti-inflammatory drugs (NSAIDs) or the use of corticosteroids but since adverse effects including gastrointestinal ulcers, hypertension, renal toxicity, and cardiovascular risks are associated with these drugs for

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201800269

pain management and inflammation,[7] finding alternatives which retain anti-inflammatory potency but with limited side effects is a high priority.

Traditional medicine plays an important role in the healthcare system of Vietnam.[8] The World Health Organization (WHO) estimates that approximately 30% of the Vietnamese population receives treatment with traditional medicines which is heavily influenced by traditional Chinese medicine.[9] *Melodorum fruticosum* Lour. (Annonaceae), which is also known in Vietnamese traditional medicine as Dũ dẻ trơn, is widely distributed in Southeast Asia,[10] and more specifically indigenous to Vietnam, Laos, Cambodia, and Thailand. The plant has edible fruits, the flowers are fragrant and are used to make perfume, while dried flowers are slightly heart stimulating and in Thailand these are used as a blood tonic. In Vietnam, the leaves are boiled in water to aid digestion.[11] Antifungal, antioxidant, and cytotoxic activities of bioactive components such as essential oils, heptenes, and butenolides isolated from the flowers and bark of *M. fruticosum* have previously been reported,[10,12–17] while there has only been one report of anti-inflammatory activity of a phenolic amide isolated from a methanolic leaf extract.[18]

To the best of our knowledge, there is no ethnopharmacological literature describing the usage of *M. fruticosum* as an anti-inflammatory agent. However, since the dichloromethane leaf extract of the plant showed promising anti-inflammatory activity in an initial screening of plants used in Vietnamese traditional medicine (results not shown), the present study aimed to identify and isolate the main components of the bioactive extract and to perform pharmacological testing on the isolated compounds by means of inhibition of interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), reactive oxygen species (ROS), and adhesion molecules (CD11b and CD62L) for attenuation of pro-inflammatory function of human neutrophils.

Results and Discussion

Extraction and Bioactivity Localization

In the present study, the anti-inflammatory potential of *M. fruticosum* leaf extracts was investigated. First, the plant material was extracted successively with dichloromethane and methanol. The obtained extracts were analyzed on their ability to inhibit the release of IL-8 in lipopolysaccharide (LPS) stimulated human neutrophils. The dichloromethane extract was highly active with 88% inhibition at a concentration of 10 μg/mL compared to the stimulated control (0% inhibition), whereas the methanol extract showed only weak inhibition (10%) at 10 μg/mL. To ensure that the inhibitory effect on IL-8 release is not due to cytotoxicity, the extracts were initially investigated on potential cytotoxicity on neutrophils. Neither the dichloromethane nor the methanol extract showed any cytotoxic effects on human neutrophils at the investigated concentration (10 μg/mL). Therefore, the bioactive dichloromethane extract was chosen for further phytochemical and pharmacological investigations. It was separated by silica gel column chromatography (CC) to obtain 47 fractions (a1–a47), which were pooled according to their compound spectra and forwarded to pharmacological testing on the release of IL-8 in human neutrophils for activity localization (results not shown). While most of the fractions were either inactive or weakly active, fraction a31 showed strong inhibitory activity on IL-8 release.

Isolation and Identification of Natural Products

Aiming at identifying the active principle of the bioactive dichloromethane extract, the crude extract and in particular the highly active fraction a31 was investigated in more detail. Analysis of fraction a31 using high performance liquid chromatography (HPLC) revealed the presence of one major compound. Purification of this compound using Sephadex® CC yielded a white powder (compound **1**). In addition to this major compound, 13 other constituents of the bioactive extract (*Figure 1*) could be isolated from various fractions by means of a combination of chromatographic techniques, i.e., silica gel and Sephadex® CC as well as semi-preparative HPLC. The isolated natural products were identified as melodamide A (**1**),[18] 4′,7-dihydroxy-5-methoxyflavanone (**2**),[19,20] haplamide (**3**),[21,22] 4′-hydroxy-5,7-dimethoxyflavanone (**4**),[23] (2*S*)-5-hydroxy-4′,7-dimethoxyflavanone (**5**),[24] 7-hydroxy-4′,5-dimethoxyflavanone (**6**),[25] 7-hydroxy-5-methoxyflavanone (**7**),[26] *N-trans*-cinnamoyltyramine (**8**),[27] (2*S*)-4′,5,7-trimethoxyflavanone (**9**),[28] 2′,4′-dihydroxy-4,6′-dimethoxychalcone (**10**),[29] 2′,4′-dihydroxy-4,6′-dimethoxydihydrochalcone (**11**),[30] and 2′,6′-dihydroxy-4′-methoxychalcone (**12**)[31] by NMR, MS, and comparison of the spectral data with those in literature. In addition, two new natural products were identified as 5-hydroxy-6-(2-hydroxybenzyl)-4′,7-dimethoxyflavanone (**13**) and 2′,4′-dihydroxy-3′-(2-hydroxybenzyl)-4,6′-dimethoxychalcone (**14**) (*Figure 2*).

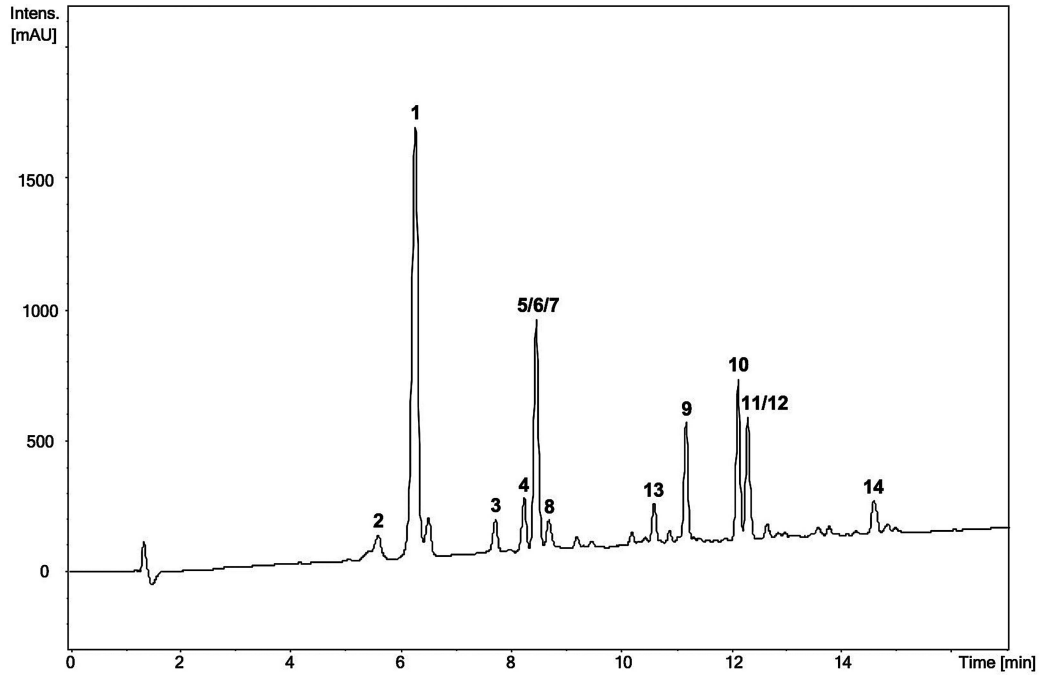

Figure 1. HPLC analysis of the dichloromethane extract of *Melodorum fruticosum*. HPLC conditions: stationary phase: Phenomenex Luna phenyl hexyl (150×4.6 mm, 5 μm particle size); mobile phase: solvent A, water; solvent B, acetonitrile; detection: DAD 230 nm, temperature: 40 °C; injection volume: 10 μL; flow rate: 1 mL/min; gradient: 0 min, 20% B; 20 min, 98% B; 30 min, 98% B. The last 13 min of the analysis are not shown.

Compounds **5** and **9** turned out to be pure enantiomers. Their assignment was based on experimental optical rotation data and their comparison to literature data from Abbate et al.[32] who used vibrational circular dichroism (VCD) to determine that the (*R*)-enantiomer of naringenin is the one exhibiting (+) optical rotation, while the (*S*)-enantiomer exhibits (−) optical rotation. Specific optical rotations ($[\alpha]_D^{20}$) of compounds **5** and **9** were $[\alpha]_D^{20} = -1.90$ ($c = 0.03$, MeOH) and $[\alpha]_D^{20} = -0.04$ ($c = 0.225$, MeOH), respectively. Therefore, they were identified as (2*S*)-5-hydroxy-4′,7-dimethoxyflavanone (**5**) and (2*S*)-4′,5,7-trimethoxyflavanone (**9**). Compounds **2**, **4**, **6**, **7**, and **13** were isolated as mixtures of (*R*)- and (*S*)-enantiomers.

Out of the 14 isolated constituents, **1**, **2**, **6**, and **12** have previously been reported from *M. fruticosum*.[18] **8**, **10**, and **11** have been reported as constituents of another *Melodorum* species, namely *M. siamensis*.[33] **3**,[21,22,34] **4**,[23] **5**,[24] **7**,[26] and **9**[28] have been reported as natural products but have not previously been isolated from *Melodorum* species. This is the first reported isolation of compounds **13** and **14**.

Structure Elucidation of New Compounds

Compound **13** was obtained as colorless solid. The HR-ESI-MS spectrum displayed a quasimolecular ion at *m/z* 405.1298 ($[M-H]^-$) consistent with the chemical formula $C_{24}H_{22}O_6$. The UV spectrum showed adsorptions at λ_{max} 204, 281, and 319 nm and the IR spectrum indicated free hydroxy (ν_{max} 3039 cm^{-1}) and ketone (ν_{max} 1604 cm^{-1}) groups. The ^{1}H-NMR spectrum of compound **13** showed two doublets at δ(H) 7.25 (d, $J = 8.7$, H(2′), H(6′)) and δ(H) 6.87 (d, $J = 8.8$, H(3′), H(5′)) consistent with a *p*-substituted aromatic ring (ring B). The singlet at δ(H) 6.18 (s, H(8)) indicated a penta-substituted aromatic ring (ring A). These signals along with the signals from the α-carbon at δ(H) 2.89 (dd, $J = 16.4$, 12.1, H(3)) and δ(H) 2.65 (dd, $J = 16.4$, 3.2, H(3)) together with the signal from the β-carbon at δ(H) 5.36 (dd, $J = 12.0$, 3.0, H(2)) from ring C confirmed that the core structure of **13** was a flavanone. Substitution of two methoxy groups was confirmed with signals at δ(H) 3.74 (3H, s) and δ(H) 3.73 (3H, s). The position of the two methoxy groups were determined from the HMBC data which revealed correlations between the proton signal of 4′-MeO (δ(H) 3.73) and C(4′) (δ(C) 158.97). The HMBC between the

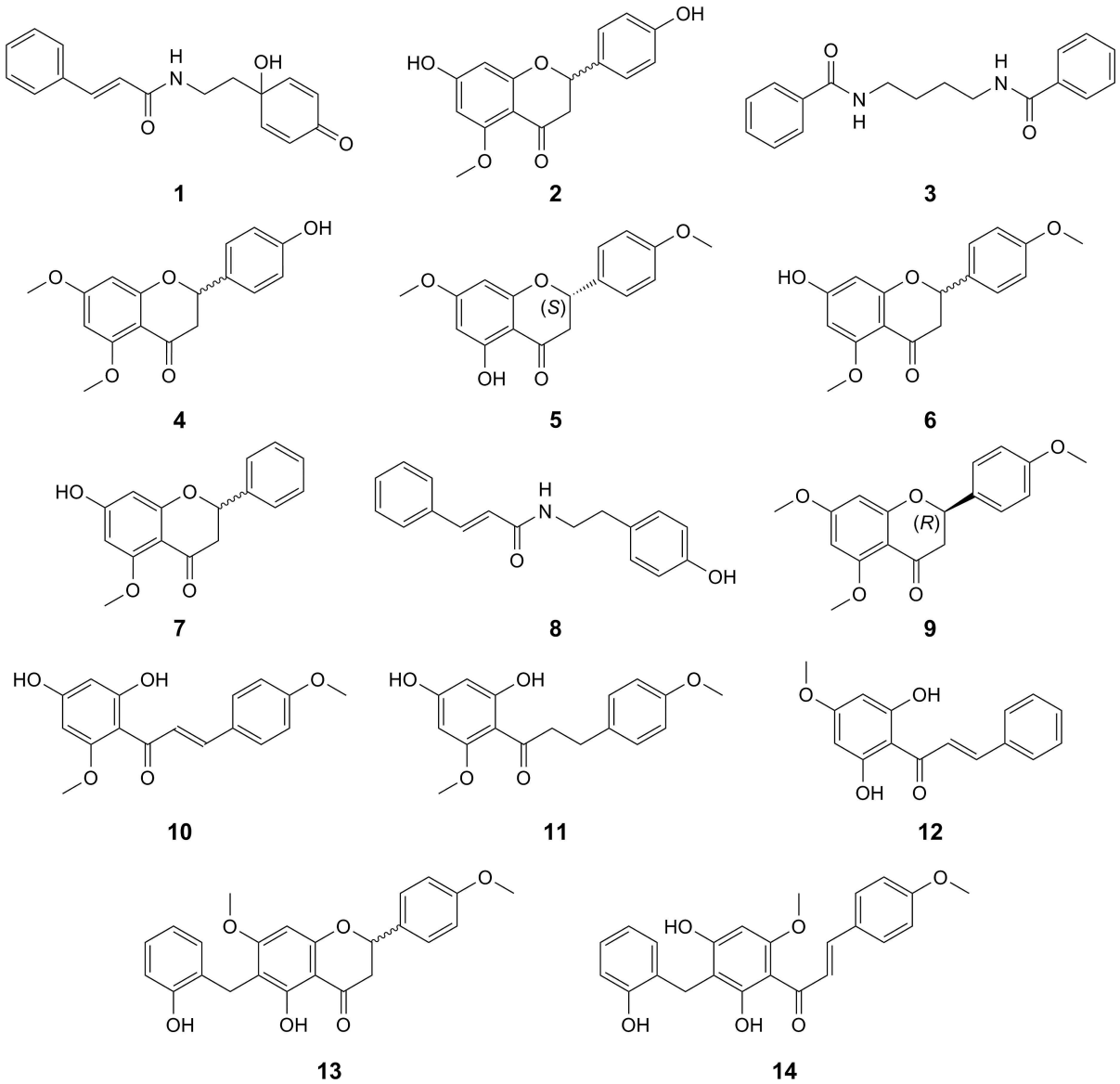

Figure 2. Chemical structures of compounds **1**–**14**.

proton signal of 7-MeO (δ(H) 3.74) and C(7) (δ(C) 160.02) and a long range COSY correlation between the proton signal of 7-MeO (δ(H) 3.74) and the proton signal of H(8) at δ(H) 6.18 confirms the position of the second methoxy attached to C(7). Further aromatic proton signals, which suggested a further aromatic ring, were observed at δ(H) 6.93 (ddd, J=8.0, 7.9, 1.8, H(4″)), δ(H) 6.74 (d, J=7.9, H(3″)), δ(H) 6.64 (d, J=6.6, H(6″)), and δ(H) 6.60 (ddd, J=7.5, 7.4, 1.1, H(5″)). Proton signals from a CH_2 group at δ(H) 3.72 (s, H(7″)) with corresponding HMBCs to the carbon (δ(C) 106.07) at position 6 of ring A and the carbon (δ(C) 126.72) at position 1″ of the additional aromatic ring indicated that the substitution of ring A by an aromatic moiety was bridged over a CH_2 group. Therefore, compound **13** was identified as 5-hydroxy-6-(2-hydroxybenzyl)-4′,7-dimethoxyflavanone.

Compound **14** was obtained as yellow solid. The HR-ESI-MS spectrum displayed a quasimolecular ion at *m/z* 405.1335 ([M−H]$^-$) consistent with the chemical formula $C_{24}H_{22}O_6$. The UV spectrum showed adsorptions at λ_{max} 202, 282, and 370 nm. The IR spectrum indicated free hydroxy (ν_{max} 3358 cm^{-1}) and ketone (ν_{max} 1602 cm^{-1}) groups. The ^{1}H-NMR spectrum of compound **14** showed two doublets at δ(H) 7.57 (d, J=8.7, H(2), H(6)) and δ(H) 6.95 (d, J=8.8, H(3), H(5)), which are consistent with a *p*-substituted aromatic ring (ring B) and a singlet at δ(H) 6.07 (s, H(5′)) for a penta-

substituted aromatic ring (ring A). These signals along with the signals from the alkene β-carbon δ(H) 7.83 (d, $J=15.5$, H(β)) and the α-carbon δ(H) 7.69 (d, $J=15.5$, H (α)) confirmed a chalcone core structure. Substitution of two methoxy groups was confirmed by signals at δ(H) 3.90 (s, 6′-MeO) and δ(H) 3.82 (s, 4-MeO). The HMBC data showed correlations between the proton signal of 4-MeO (δ(H) 3.82) and C(4) (δ(C) 163.01), and correlations between the proton of 6′-MeO (δ(H) 3.90) and C (6′) (δ(C) 162.93). Long range COSY correlations between the 6′-MeO proton at δ(H) 3.90 and the H(5′) proton at δ(H) 6.07 confirmed this position. A further long range COSY correlation between the proton of 4-MeO (δ(H) 3.82) and δ(H) 6.95 (H(3), H(5)) was observed. Aromatic proton signals, which indicated a further aromatic ring, were observed at δ(H) 7.13 (dd, $J=7.6$, 1.4, H(6″)), δ(H) 7.00–6.96 (m, H(4″)), δ(H) 6.76 (dd, $J=$ 8.0, 1.0, H(3″)), and δ(H) 6.70 (td, $J=7.5$, 1.1, H(5″)). Proton signals from a CH_2 group at δ(H) 3.84 (s, H(7″)) with corresponding HMBCs to the carbon (δ(C) 108.52) at position 3′ of ring A and the carbon (δ(C) 127.88) at position 1″ of the additional aromatic ring indicated that the substitution of ring A by an aromatic moiety was bridged over a CH_2 group. The NMR shifts were compared to those described by Prawat et al.[33] who isolated a similar chalcone, whereby the α and β carbons were connected by a single bond rather than a double bond as it is the case in compound **14**. Therefore, compound **14** was identified as 2′,4′-dihydroxy-3′-(2-hydroxybenzyl)-4,6′-dimethoxychalcone.

Biological Activities of Isolated Compounds

To identify the constituents responsible for the IL-8 inhibition activity of the dichloromethane extract of the *M. fruticosum* leaves, all isolated compounds were tested on their ability to inhibit IL-8 release in human neutrophils. In particular, cells were stimulated with LPS to activate an inflammatory response whereby the levels of IL-8 were measured by ELISA. Quercetin (10 μM), which was used as a positive control, decreased the release of IL-8 from neutrophils to 50.9 ± 3.5% compared to the stimulated control (100% IL-8 production). At the same concentration (10 μM), compounds **1**, **10**, and **12** also showed statistically significant ($p<0.001$) decreases of IL-8 release (6.1 ± 0.5, 73.4 ± 3.4, and 30.4 ± 2.3% IL-8 production, respectively; *Figure 3*a). Since the observed activities of these compounds were in the range of the activity of the positive control, the active compounds **1**, **10**, and **12** were analyzed in more detail in concentration response studies. All three compounds showed inhibition of IL-8 production in a concentration dependent manner with the IC_{50} values of 6.6, 8.6, and 11.6 μM, respectively (*Figure 3*b).

Resolution of inflammation can be achieved by decreasing levels of pro-inflammatory mediators and/or hindering the recruitment of leukocytes and their activation.[35] IL-8 is an important pro-inflammatory chemokine produced by neutrophils and acts as a mediator of inflammation by recruiting neutrophils to the site of inflammation.[36] Another important pro-inflammatory cytokine, which has been identified as a key regulator of inflammatory processes, is TNF-α. Therapeutic blockade of TNF-α has been shown to be highly beneficial in the treatment of chronic inflammatory conditions.[37–39]

Therefore, the effects of the isolated compounds on the TNF-α production by LPS stimulated human neutrophils were also determined using an ELISA assay. Compounds **1**, **10**, and **12** at 10 μM showed pronounced inhibitory effects on TNF-α production of LPS stimulated human neutrophils (*Figure 4*a). The inhibition of TNF-α production was concentration dependent as shown in *Figure 4*b. The IC_{50} values of **1**, **10**, and **12** reached 4.5, 13.3, and 6.2 μM, respectively (the IC_{50} value of the positive control quercetin: 10 μM).

Stimulation of human neutrophils results in the rapid phosphorylation of proteins, including p38 mitogen-activated protein kinase (MAPK), p42/44 extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and Akt kinase.[40–42] Therefore, the decrease in the release of IL-8 as well as of TNF-α by compounds **1**, **10**, and **12** might be related to MAPK activation inhibition. The study of Chan et al. revealed that melodamide A (**1**) inhibits the p38 MAPK pathway without affecting p42/44 and Akt kinases phosphorylation.[18] However, other mechanisms of action, such as NF-κB activation inhibition, might be involved.

To ensure that the observed inhibitory effects on IL-8 and TNF-α release by the active compounds **1**, **10**, and **12** are not due to cytotoxicity, they were additionally analyzed for potential cytotoxic effects. This could be excluded since at the investigated concentration (10 μM) the three compounds did not show any cytotoxic effect on neutrophils (results not shown).

Reactive oxygen species (ROS) are essential in the progression of inflammation, acting both as signaling molecules and as mediators of inflammation.[1] Therefore, it was also evaluated whether the isolated compounds inhibit ROS production in *N*-formylmethionyl-leucyl-phenylalanine (f-MLP) stimulated neu-

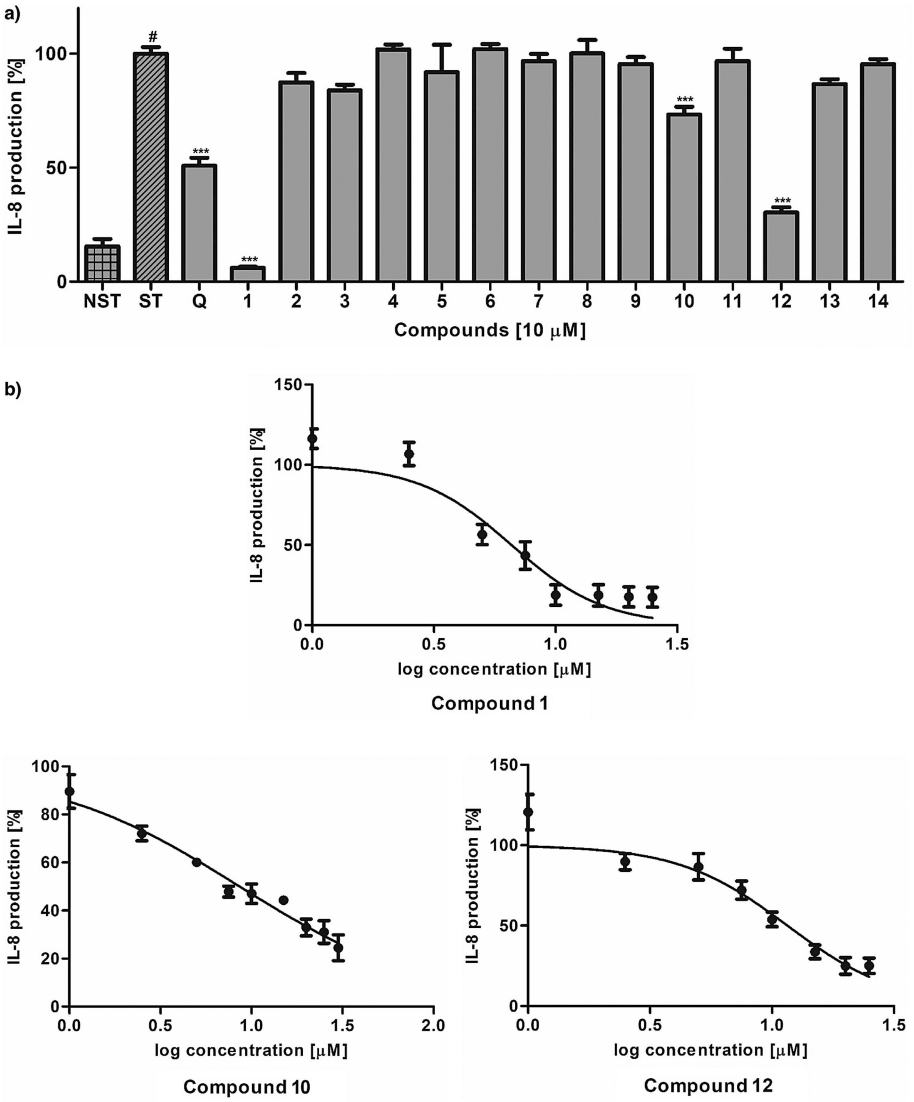

Figure 3. Inhibition of IL-8 production in LPS stimulated human neutrophils, given as % of stimulated control (100%). a) Inhibition by compounds **1**–**14** at a concentration of 10 μM. b) Concentration dependent inhibition of IL-8 production by compounds **1**, **10**, and **12**. Results shown are the mean ± SEM of three independent experiments, assayed in triplicate. Experiments were performed using cells from different donors. Statistical significance: # $p < 0.001$ compared to non-stimulated control, *** $p < 0.001$ compared to stimulated control (Dunnett's post hoc test). ST stimulated control, NST non-stimulated control. Quercetin (Q) was used as a positive control (10 μM).

trophils. The incubation of stimulated neutrophils with compounds **1**–**14** at 10 μM resulted in a statistically significant decrease in ROS production by compounds **4**, **6**, and **13** to 47.7 ± 5.6, 48.8 ± 5.9, and 48.3 ± 2.6%, respectively, when compared to the stimulated cells (100% ROS production). However, they were less active when compared to the positive control quercetin (10 μM), which decreased ROS production to 28.5 ± 5.7%. Interestingly, all other compounds, including **1**, **10**, and **12**, showed no significant inhibitory activity on ROS production (*Figure 5*).

The interaction of leukocytes with vascular endothelial cells is crucial in inflammatory and immune responses. Adhesion molecules, which are expressed on the surface of neutrophils, are key mediators allowing for an accumulation of leukocytes at the site of inflammation. Several adhesion molecules are part of this process such as selectins, responsible for the recruitment of leukocytes to the site of inflammation, and integrins which are involved in adhesion of leukocytes to the endothelial cells.[43] When neutrophils are stimulated with pro-inflammatory LPS, down-

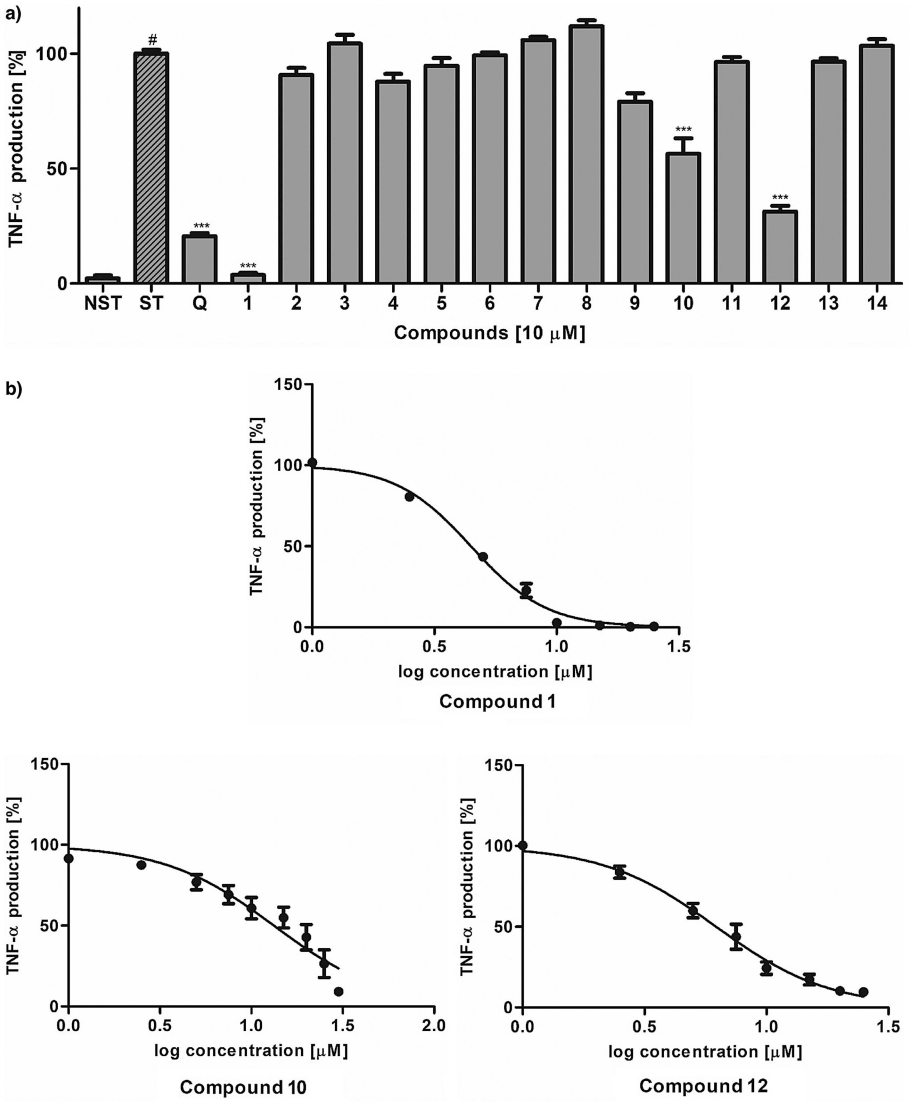

Figure 4. Inhibition of TNF-α production in LPS stimulated human neutrophils, given as % of stimulated control (100%). a) Inhibition by compounds **1**–**14** at a concentration of 10 μM. b) Concentration dependent inhibition of TNF-α production by compounds **1**, **10**, and **12**. Results shown are the mean ± SEM of three independent experiments, assayed in triplicate. Experiments were performed using cells from different donors. Statistical significance: # $p < 0.001$ compared to non-stimulated control, *** $p < 0.001$ compared to stimulated control (Dunnett's post hoc test). ST stimulated control, NST non-stimulated control. Quercetin (Q) was used as a positive control (10 μM).

regulation of the L-selectin CD62L and upregulation of the integrin CD11b occur. A compound which can inhibit firm adhesion and extravascular migration to the site of inflammation is thus able to alleviate local inflammation.[44] Therefore, within this study, the effects of the isolated compounds on CD11b and CD62L expression in stimulated neutrophils were analyzed. However, no inhibition of CD11b surface expression triggered by cytochalasin A/f-MLP was observed for compounds **1**–**14** at a concentration of 10 μM when compared to the stimulated control (*Figure 6*). Results depicted in *Figure 7* show that compounds **1**–**14** at a concentration of 10 μM also did not prevent CD62L from shedding (values compared to stimulated control) after stimulation with cytochalasin A/f-MLP.

Although the use of *M. fruticosum* as a digestive aid and blood tonic in traditional medicine has been reported,[11] there is no mention of any specific anti-inflammatory application to which the activity of the dichloromethane extract or isolated compounds could be correlated. However, the anti-inflammatory

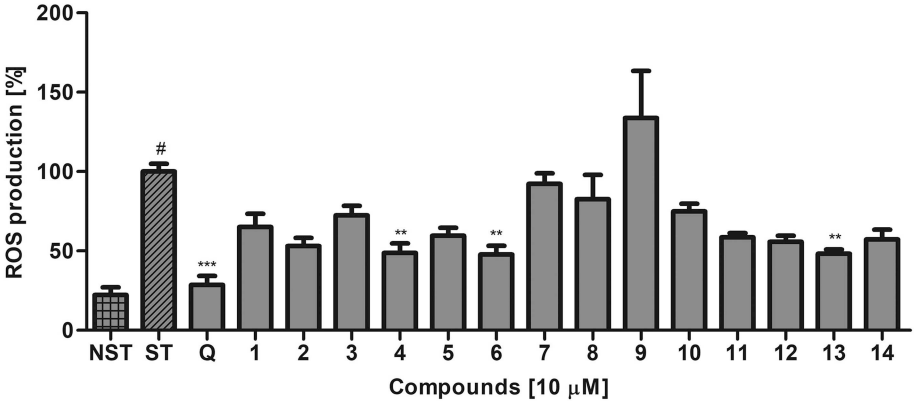

Figure 5. Inhibition of ROS production in f-MLP stimulated human neutrophils by compounds **1**–**14** at a concentration of 10 μM, given as % of stimulated control (100%). Results shown are the mean ± SEM of three independent experiments, assayed in triplicate. Experiments were performed using cells from different donors. Statistical significance: # $p < 0.001$ compared to non-stimulated control, *** $p < 0.001$ and ** $p < 0.05$ compared to stimulated control (Dunnett's post hoc test). ST stimulated control, NST non-stimulated control. Quercetin (Q) was used as a positive control (10 μM).

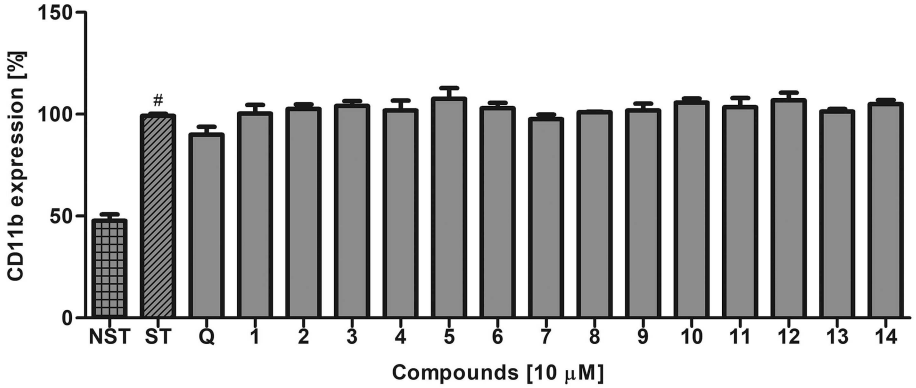

Figure 6. Effect of compounds **1**–**14** at a concentration of 10 μM on CD11b expression in cytochalasin A/f-MLP stimulated human neutrophils, given as % of stimulated control (100%). Results shown are the mean ± SEM of three independent experiments using cells from different donors. Statistical significance: # $p < 0.001$ compared to non-stimulated control. ST stimulated control, NST non-stimulated control. Quercetin (Q) was used as a positive control (50 μM).

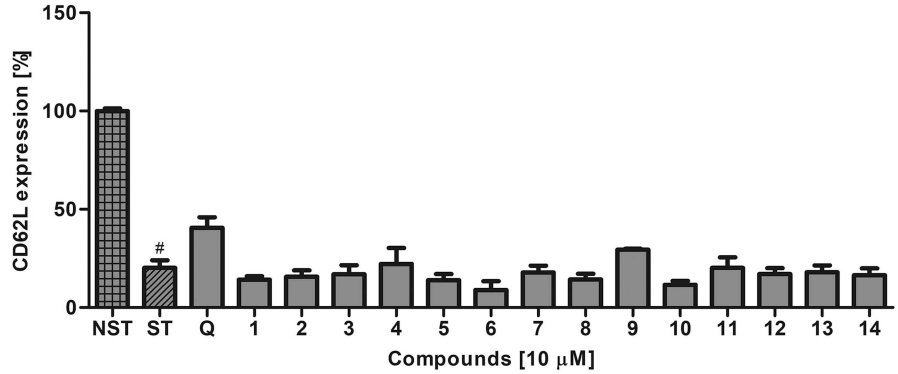

Figure 7. Effect of compounds **1**–**14** at a concentration of 10 μM on CD62L expression in cytochalasin A/f-MLP stimulated human neutrophils, given as % of non-stimulated control. Results shown are the mean ± SEM of three independent experiments using cells from different donors. Statistical significance: # $p < 0.001$ compared to non-stimulated control. ST stimulated control, NST non-stimulated control. Quercetin (Q) was used as a positive control (50 μM).

activity of compound **1** has been reported previously by Chan et al. who isolated it from the methanolic extract of the leaves of *M. fruticosum*. In particular, they reported the inhibition of superoxide anion generation in f-MLP/cytochalasin B activated human neutrophils by compound **1** with an IC_{50} value of 5.25 μM, which could be attributed to inhibition of the p38 mitogen-activated protein kinases (MAPK) signaling pathway. Further substitutions with halide, NO_2, MeO, and Me groups on ring A of **1** did not further increase the anti-inflammatory activity.[18]

Interestingly, all of the active compounds (**1**, **10**, and **12**), being able to inhibit the release of IL-8 and TNF-α production, possess either an α,β-unsaturated carbonyl or an α,β-unsaturated amide moiety. Michael acceptor systems such as these are able to inhibit cysteine proteases by forming covalent bonds with the active site thiol of cysteine proteases.[45] Harikumar et al. suggested that the prenylated chalcone xanthohumol, with one Michael acceptor and an electrophilic carbonyl group, interacts directly with the sulfhydryl group of the cysteine residue of IκBα kinase (IKK) and the p65 subunit of nuclear factor-κB (NF-κB), leading to an adduct formation and therefore inhibiting the NF-κB activation pathway.[46] Further enhancement of chalcone thiol activity by modifying the α-position of the α,β-unsaturated carbonyl with strong electrophiles has also been reported.[47] However, undesired toxic effects can also occur through reactions with electrophilic groups causing disruptive effects of biological macromolecules.[48,49] Michael reactivity is strongly influenced by the substituents on the α- and β-carbons, whereby electron attracting groups increase reactivity while electron donating groups reduce activity.[50] A recent perspective by Jackson et al. focused on the reactivity of biological thiols with Michael acceptors concluding that the reactivity decreases in the order of enals > enones > α,β-unsaturated esters > acrylamides > α,β-unsaturated carboxylic acids.[51] For the reactivity of chalcones, the presence of a hydroxy group at the 2′ position is crucial, whereby activation of the carbonyl group through the intramolecular H-bond and stabilization of the conjugation in the system occurs.[52] The activity of the chalcones **10** and **12**, in which a nucleophilic attack is possible owing to the Michael acceptor, may be explained by this type of interaction, since the dihydrochalcone **11**, which lacks this important Michael acceptor site, did not show any activity. This could also be the mechanism of action for compound **1** owing to the α,β-unsaturated amide moiety. However, further studies are necessary to confirm this hypothesis. Furthermore, since compounds, which alkylate cysteinyl thiols via Michael addition, often possess undesired toxic effects, the identified bioactive compounds should be analyzed in further toxicity studies to exclude potential cytotoxicity for humans, even though the traditional usage of *M. fruticosum* as a medicinal plant in Vietnam suggests the lack of severe toxicity.

Although compounds **1**, **10**, and **12** actively inhibited the release of IL-8 and TNF-α, no inhibition of CD11b surface expression or CD62L shedding was observed. This observation is similar to those by Arshad et al. who investigated a series of α,β-unsaturated carbonyl-based compounds, namely curcumin analogs and chalcone derivatives, and although moderate to strong activity for migration of neutrophils, phagocytic, and ROS production was observed, less activity was observed for CD11b/CD18 expression of leukocytes.[53] The interaction target of the active compounds **1**, **10**, and **12** appears to be specific to cytokine production rather than the interaction of leukocytes with vascular endothelial cells.

Conclusions

The anti-inflammatory properties of the Vietnamese traditional medicinal plant *M. fruticosum* presented within this study could not be supported by any documented ethnopharmacological survey known to date. However, the initial bioactivity of the active dichloromethane *M. fruticosum* leaf extract could be traced back to the presence of compounds **1**, **10**, and **12**, of which compound **1** represents a major constituent of the bioactive extract. The results suggest that the mode of action for compounds **1**, **10**, and **12**, which actively inhibited release of IL-8 and TNF-α, is specifically attributed to those mechanisms rather than the generation of ROS or the interaction with other functions of leukocytes such as rolling, firm adhesion, or degranulation at the site of inflammation. The identification of the anti-inflammatory effects of *M. fruticosum* might provide a basis for potential future usage of the plant against inflammatory conditions.

Experimental Section

General

HPLC analysis was performed on a HP 1100 system (Agilent, Waldbronn, Germany) equipped with an autosampler, column thermostat, and DAD detector;

stationary phase: Phenomenex Luna phenyl hexyl (150×4.6 mm, 5 μm particle size); mobile phase: solvent A, water; solvent B, acetonitrile; detection: DAD 230 nm, temperature: 40 °C; injection volume: 10 μL; flow rate: 1 mL/min; gradient: 0 min, 20% B; 20 min, 98% B; 30 min, 98% B. Optical rotations were determined with a PerkinElmer 341 polarimeter (Wellesley, MA, USA) at 20 °C. The ultraviolet spectra were recorded on a Thermo Scientific Multiskan GO spectrophotometer (Waltham, MA, USA); λ_{max} (log ε) in nm. IR spectra were recorded on a Bruker ALPHA spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a PLATINUM-ATR unit (spectral range 4000–400 cm^{-1}, 4 scans per cm^{-1}, OPUS 7 software); $\tilde{\nu}$ in cm^{-1}. NMR spectra (1D- and 2D-experiments) were acquired on a Bruker Advance II 600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600 MHz (^1H) and 151 MHz (^{13}C) at 300 K (chemical shifts δ in ppm, coupling constants J in Hz). NMR solvents used were CD_3OD or $(D_6)DMSO$ with 0.03% TMS (Eurisotop, Gif-Sur-Yvette, France) as an internal standard. Mass spectrometry was performed on an Esquire 3000^{plus} (Bruker Daltonics, Bremen, Germany) with the following parameters: ESI; temperature: 325 °C; dry gas: 8.00 L/min; nebulizer: 30 psi; full scan mode: *m/z* 50–1500. HR-ESI-MS data were obtained using a Bruker mikrOTOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany); in *m/z*.

Plant Material

Leaves from *M. fruticosum* were collected in the Tam thành commune, Phú ninh district, Quàng Nam Province of Vietnam in August 2014 and identified by Dr. Võ Văn Chi from the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City. A voucher specimen (BW-20141210-03) of the air-dried plant material was deposited at the Institute of Pharmacy/Pharmacognosy of the University of Innsbruck.

Chemicals and Reagents

All solvents used for isolation were purchased from VWR International (Darmstadt, Germany). Solvents used for HPLC were purchased from Merck (Darmstadt, Germany) and ultrapure water was produced onsite by a Sartorius Arium 611 UV water purification system (Göttingen, Germany). Luminol, f-MLP, LPS, cytochalasin A, Hanks' balanced salt solution (HBSS), RPMI 1640 medium, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and L-glutamine were purchased from Sigma–Aldrich GmbH (Steinheim, Germany). Quercetin (95% purity) was purchased from Carl Roth (Karlsruhe, Germany). Propidium iodide was purchased from BD Biosciences (San Diego, CA, USA). Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco (Carlsbad, CA, USA). Both CD62L-(APC)-conjugate and CD11b-(PE)-conjugate were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Preparation of Extracts for Activity Screening

Air-dried and ground *M. fruticosum* leaf material (5 g) was extracted with 30 mL dichloromethane by sonication for 10 min at room temperature. The plant material was recovered by filtration and the extraction process was repeated twice with 20 mL of dichloromethane, respectively. The filtrates were combined and the solvent was evaporated under reduced pressure to yield 236.9 mg of crude dichloromethane extract. The residual plant material was air-dried and the extraction process was repeated as described with methanol to yield 677.17 mg of crude methanol extract. Both extracts were subjected to pharmacological testing on their inhibitory effects on the release of IL-8 in human neutrophils.

Preparation of the Extract for Compound Isolation

Powdered dried leaves of *M. fruticosum* (952 g) were exhaustively extracted with dichloromethane by sonication and the solvent was evaporated under reduced pressure to yield 63.8 g of crude extract.

Fractionation and Compound Isolation

The crude dichloromethane extract (30 g) was subjected to silica gel CC (605 g of silica gel 60, grain size 40–63 μm, Merck, Darmstadt, Germany), eluting with a stepwise gradient of dichloromethane, ethyl acetate, and methanol. The eluate was monitored by TLC and combined to yield 47 fractions (fractions a1–a47), which were pooled according to their compound spectra and forwarded to pharmacological testing on the release of IL-8 in human neutrophils for activity localization (results not shown).

Fraction a8 (216 mg) was separated by Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v) as mobile phase to yield compound **14** (6.75 mg). Fraction a9 (219 mg) was also subjected to Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v)

as mobile phase to yield compound **12** (12.34 mg). Separation of fraction a10 (237 mg) using Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v) as mobile phase led to the isolation of compounds **10** (66.84 mg) and **11** (19.91 mg), respectively. Fraction a11 (402.3 mg) was first separated by Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v) as mobile phase to yield seven sub-fractions (a11.b1–a11.b7). Separation of sub-fraction a11.b1 (330 mg) using semi-preparative HPLC (column: Phenomenex Synergi polar RP, 250×10 mm, 80 Å, 4 μm particle size; detection: DAD 230 nm; temperature: 40 °C; mobile phase: water (solvent A), acetonitrile (solvent B); gradient composition: 0 min, 40% B; 8 min, 80% B; 12.5 min, 98% B; flow rate: 2 mL/min) led to the isolation of compound **9** (5.74 mg). Fraction a16 (202 mg) was separated by Sephadex® LH-20 with dichloromethane/acetone (85:15, v/v) as mobile phase to yield compounds **4** (37.84 mg), **7** (15.55 mg), and **8** (7.79 mg), respectively. Fraction a21 (201 mg) was separated by Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v) as mobile phase to give eight sub-fractions (a21.b1–a21.b8). Sub-fraction a21.b7 (24.68 mg) was further separated by semi-preparative HPLC (column: Phenomenex Synergi polar RP, 250×10 mm, 80 Å, 4 μm particle size; detection: DAD 230 nm; temperature: 40 °C; isocratic 50% water/50% acetonitrile, 17 min; flow rate: 2 mL/min) to yield compounds **6** (11.51 mg) and **13** (4.24 mg), respectively. Fraction a26 (171 mg) was separated by Sephadex® LH-20 CC with methanol as mobile phase to give eight sub-fractions (a26.b1–a26.b8). Sub-fraction a26.b7 (10.72 mg) was subjected to semi-preparative HPLC (column: Phenomenex Synergi polar RP, 250×10 mm, 80 Å, 4 μm particle size; detection: DAD 230 nm; temperature: 40 °C, isocratic 50% water/50% acetonitrile, 15 min; flow rate: 2 mL/min) to yield compounds **2** (6.07 mg) and **5** (3.90 mg), respectively. Fraction a29 (205 mg) was separated by Sephadex® LH-20 CC with dichloromethane/acetone (85:15, v/v) as mobile phase to yield compound **3** (16.74 mg). Fraction a31 (208 mg), which was the most active fraction, was separated by Sephadex® LH-20 CC with methanol as mobile phase to yield compound **1** (19.19 mg). Purity was determined by HPLC-DAD analysis at 205 nm and calculated as >95% for all isolated compounds.

5-Hydroxy-6-(2-hydroxybenzyl)-4′,7-dimethoxyflavanone (**13**). Colorless solid. $[\alpha]_D^{20} = -1.90$ (c = 0.03, MeOH). UV (MeOH): 204 (8.13), 281 (3.72), 319 (3.52). IR (ATR): 3039, 2918, 2849, 2724, 2615, 1337, 1604, 1567, 1515, 1488, 1455, 1410, 1354, 1327, 1283, 1247, 1201, 1174, 1146, 1101, 1083, 1032, 967, 933, 893, 874, 826, 782, 747, 697, 643, 616, 598, 577, 548, 535, 451, 418. ^{1}H-NMR (600 MHz, (D_6)DMSO): 7.25 (d, J = 8.7, H(2′), H(6′)), 6.93 (ddd, J = 8.0, 7.9, 1.8, H(4″)), 6.87 (d, J = 8.8, H(3′), H(5′)), 6.74 (d, J = 7.9, H(3″)), 6.64 (d, J = 6.6, H(6″)), 6.60 (ddd, J = 7.5, 7.4, 1.1, H(5″)), 6.18 (s, H(8)), 5.36 (dd, J = 12.0, 3.0, H(2)), 3.74 (s, 7-MeO), 3.73 (s, 4′-MeO), 3.72 (s, H(7″)), 2.89 (dd, J = 16.4, 12.1, H(3)), 2.65 (dd, J = 16.4, 3.2, H(3)). ^{13}C-NMR (151 MHz, (D_6)DMSO): 187.86 (C(4)), 161.76 (C(5), C(9)), 160.02 (C(7)), 158.97 (C(4′)), 154.93 (C(2″)), 131.25 (C(1′)), 127.81 (C(6″)), 127.43 (C(2′), C(6′)), 126.72 (C(1″)), 126.11 (C(4″)), 118.48 (C(5″)), 114.42 (C(3″)), 113.67 (C(3′), C(5′)), 106.07 (C(6)), 104.45 (C(10)), 92.94 (C(8)), 77.35 (C(2)), 55.29 (7-MeO), 55.03 (4′-MeO), 44.42 (C(3)), 22.00 (C(7″)) (see *Supporting Information*).

2′,4′-Dihydroxy-3′-(2-hydroxybenzyl)-4,6′-dimethoxychalcone (**14**). Yellow solid. UV (MeOH): 202 (4.05), 282 (3.28), 370 (3.74). IR (ATR): 3358, 2961, 2923, 2850, 1602, 1510, 1488, 1454, 1422, 1346, 1290, 1258, 1235, 1172, 1138, 1106, 1052, 1031, 980, 866, 826, 800, 755. ^{1}H-NMR (600 MHz, CD_3OD): 7.83 (d, J = 15.5, H(β)), 7.69 (d, J = 15.5, H(α)), 7.57 (d, J = 8.7, H(2), H(6)), 7.13 (dd, J = 7.6, 1.4, H(6″)), 7.00–9.96 (m, H(4″)), 6.95 (d, J = 8.8, H(3), H(5)), 6.76 (dd, J = 8.0, 1.0, H(3″)), 6.70 (td, J = 7.5, 1.1, H(5″)), 6.07 (s, H(5′)), 3.90 (s, 6′-MeO), 3.84 (d, J = 1.7, H(7″)), 3.82 (s, 4-MeO). ^{13}C-NMR (151 MHz, CD_3OD): 193.94 (C=O), 166.24 (C(2′), 164.55 (C(4′)), 163.01 (C(4)), 162.93 (C(6′)), 155.52 (C(2″)), 143.13 (C(α)), 131.21 (C(6″)), 131.09 (C(2), C(6)), 129.57 (C(1)), 128.48 (C(4″)), 127.88 (C(1″)), 126.51 (C(β)), 120.79 (C(5″)), 116.02 (C(3″)), 115.45 (C(5), C(3)), 108.52 (C(3′)), 106.44 (C(1′)), 92.30 (C(5′)), 56.22 (6′-MeO), 55.86 (4-MeO), 23.11 (C(7″)) (see *Supporting Information*).

Isolation of Human Neutrophils

Peripheral venous blood was taken from healthy human donors at the Warsaw Blood Donation Center. Neutrophils were isolated using a standard dextran sedimentation followed by hypotonic lysis of erythrocytes and centrifugation in a Ficoll Hypaque gradient. Neutrophils were suspended in the appropriate medium for the respective assay and stored at 4 °C prior to use.

Cytotoxicity

Cytotoxicity of the crude extracts and pure compounds was determined by standard flow cytometric

probe using propidium iodide (PI) staining to distinguish cells with diminished membrane integrity. Neutrophils (2×10^5/mL) were incubated in a 24-well plate in RPMI 1640 medium (without calcium and magnesium) with 10% FBS, 10 mM HEPES, and 2 mM L-glutamine for 24 h at 37 °C with 5% CO_2, in the presence of an extract at a concentration of 10 μg/mL or a pure compound at a concentration of 10 μM, and stimulated with 10 μL LPS (100 ng/mL). After 24 h, the neutrophils were harvested and centrifuged (1500 RPM, 10 min, 4 °C). The neutrophils were suspended in 400 μL PBS with 5 μL propidium iodide (50 μg/mL) and left in the dark for 15 min. Cells were analyzed by flow cell cytometry (BD FACSCalibur flow cytometer, BD biosciences, San Jose, CA, USA) and the data from 10,000 events were recorded. Cells, which displayed high permeability to PI, were expressed as a percentage of PI(+) cells.

IL-8 and TNF-α Production

Neutrophils were incubated in a 24-well plate in RPMI 1640 medium with 10% FBS, 10 mM HEPES, and 2 mM L-glutamine for 24 h at 37 °C with 5% CO_2, in the presence of an extract at a concentration of 10 μg/mL or a pure compound at a concentration of 10 μM, and stimulated with 10 μL LPS (100 ng/mL). Quercetin (10 μM) was used as a positive control. The amount of IL-8 or TNF-α released into the cell supernatants was measured by ELISA following the manufacturer's instructions (BD Biosciences). The effect on IL-8 or TNF-α production was calculated as a percentage of released agents in comparison with the stimulated control. IC_{50} values were determined from experiments, in which compounds **1**, **10**, and **12** were added at concentrations of 1, 2.5, 5, 7.5, 10, 15, 20, and 25 μM.

Expression of Adhesion Molecules CD62L and CD11b

Cell suspensions (400 μL) of neutrophils (1×10^6/mL) in PBS buffer were incubated with 100 μL of pure compounds (10 μM) for 30 min at 37 °C before a stimulation solution containing 60 μL f-MLP (0.1 μg/mL) and 30 μL cytochalasin A (5 μg/mL) was added. After a further incubation time of 30 min at 37 °C, neutrophils were marked with monoclonal antibody against CD62L-(APC)-conjugate and CD11b-(PE)-conjugate and left in the dark for 15 min prior to analysis by flow cell cytometry (BD FACSCalibur flow cytometer, BD biosciences, San Jose, CA, USA), on which the data from 10,000 events were recorded. Quercetin (50 μM) was used as a positive control.

Evaluation of ROS Production by Human Neutrophils

The evaluation of the ROS production by f-MPL stimulated neutrophils was determined using luminol dependent chemiluminescence. Following isolation, 70 μL of the neutrophil suspension (2×10^5/mL) in Hanks balanced salt solution was incubated with 50 μL of each compound (10 μM) together with 50 μL luminol (100 μM). ROS production was stimulated by the addition of 30 μL f-MLP (0.1 μg/mL). Chemiluminescence was measured immediately at 37 °C at 2-min intervals over a 45-min period. Quercetin (10 μM) was used as a positive control.

Statistical Analysis

GraphPad Prism (version 5.01) was used to plot data and to calculate statistical significance. The results are expressed as mean ± SEM. Nonlinear regression (log inhibitor vs. normalized response, variable slope) was used to calculate IC_{50} values. Statistical significance was determined by ANOVA with Dunnett's post hoc test. *P* values below 0.05 were considered statistically significant.

Acknowledgements

This work was supported by the EU–H2020-Marie-Skłodowska-Curie Actions (RISE project No. 691158 – 'MediHealth'), as well as the Austrian Federal Ministry of Science, Research and Economy (BMWFW), and the Polish Ministry of Science and Higher Education (MNiSW) in the frame of an Austrian–Polish cooperation agreement (project No. PL13/2015 of the Austrian Agency for International Cooperation in Education and Research, OeAD-GmbH). The authors would like to thank Prof. B. Matuszczak for IR measurements, S. Moosmang for HR-ESI-MS measurements, and D. Nguyen for translation of Vietnamese literature.

Author Contribution Statement

N. S. E. isolated and characterized the compounds and drafted the manuscript. *L. H.* collected the plant material and performed phytochemical experiments. *N. S. E.* and *B. M.* performed the biological experiments. *B. W.* and *A. K. K.* analyzed the data and supported planning and interpretation of the experiments. *H. T.*, *A. K. K.*, and *H. S.* contributed reagents, materials, and analysis tools. *B. W.*, *H. T.*, *A. K. K.*, and *H. S.* conceived

and supervised the study. All the authors approved the final manuscript and declare no conflict of interest associated with this work.

References

[1] M. Mittal, M. R. Siddiqui, K. Tran, S. P. Reddy, A. B. Malik, 'Reactive oxygen species in inflammation and tissue injury', *Antioxid. Redox Signaling* **2014**, *20*, 1126–1167.

[2] M. Faurschou, N. Borregaard, 'Neutrophil granules and secretory vesicles in inflammation', *Microbes Infect.* **2003**, *5*, 1317–1327.

[3] A. Mantovani, M. A. Cassatella, C. Costantini, S. Jaillon, 'Neutrophils in the activation and regulation of innate and adaptive immunity', *Nat. Rev. Immunol.* **2011**, *11*, 519–531.

[4] R. Baetta, A. Corsini, 'Role of polymorphonuclear neutrophils in atherosclerosis: current state and future perspectives', *Atherosclerosis* **2010**, *210*, 1–13.

[5] R. Cascão, H. S. Rosário, J. E. Fonseca, 'Neutrophils: warriors and commanders in immune mediated inflammatory diseases', *Acta Reumatol. Port.* **2009**, *34*, 313–326.

[6] J. C. Brazil, N. A. Louis, C. A. Parkos, 'The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease', *Inflammatory Bowel Dis.* **2013**, *19*, 1556–1565.

[7] M. Wehling, 'Non-steroidal anti-inflammatory drug use in chronic pain conditions with special emphasis on the elderly and patients with relevant comorbidities: management and mitigation of risks and adverse effects', *Eur. J. Clin. Pharmacol.* **2014**, *70*, 1159–1172.

[8] H. J. Woerdenbag, T. M. Nguyen, D. V. Vu, H. Tran, D. T. Nguyen, T. V. Tran, P. A. G. M. De Smet, J. R. B. J. Brouwers, 'Vietnamese traditional medicine from a pharmacist's perspective', *Expert Rev. Clin. Pharmacol.* **2012**, *5*, 459–477.

[9] World Health Organization (WHO), 'Legal status of traditional medicine and complementary/alternative medicine: a worldwide review', Geneva, Switzerland, **2001**.

[10] P. Pripdeevech, E. Chukeatirote, 'Chemical compositions, antifungal and antioxidant activities of essential oil and various extracts of *Melodorum fruticosum* L. flowers', *Food Chem. Toxicol.* **2010**, *48*, 2754–2758.

[11] V. C. Võ, 'Dictionary of Common Plants', Science and Technique Publishing House, Hà Noi, **2003**.

[12] J. H. Jung, S. Pummangura, C. Chaichantipyuth, C. Patarapanich, P. E. Fanwick, C.-J. Chang, J. L. McLaughlin, 'New bioactive heptenes from *Melodorum fruticosum* (Annonaceae)', *Tetrahedron* **1990**, *46*, 5043–5054.

[13] J. H. Jung, S. Pummangura, C. Chaichantipyuth, C. Patarapanich, J. L. McLaughlin, 'Bioactive constituents of *Melodorum fruticosum*', *Phytochemistry* **1990**, *29*, 1667–1670.

[14] J. H. Jung, C.-J. Chang, D. L. Smith, J. L. McLaughlin, S. Pummangura, C. Chaichantipyuth, C. Patarapanich, 'Additional bioactive heptenes from *Melodorum fruticosum*', *J. Nat. Prod.* **1991**, *54*, 500–505.

[15] P. Tuchinda, J. Udchachon, V. Reutrakul, T. Santisuk, W. C. Taylor, N. R. Farnsworth, J. M. Pezzuto, A. D. Kinghorn, 'Bioactive butenolides from *Melodorum fruticosum*', *Phytochemistry* **1991**, *30*, 2685–2689.

[16] C. Chaichantipyuth, S. Tiyaworanan, S. Mekaroonreung, N. Ngamrojnavanich, S. Roengsumran, S. Puthong, A. Petsom, T. Ishikawa, 'Oxidized heptenes from flowers of *Melodorum fruticosum*', *Phytochemistry* **2001**, *58*, 1311–1315.

[17] R. Mongkol, J. Piapukiew, W. Chavasiri, 'Chemical constituents from *Melodorum fruticosum* Lour. flowers against plant pathogenic fungi', *Agric. Nat. Res.* **2016**, *50*, 270–275.

[18] H.-H. Chan, T.-L. Hwang, T. D. Thang, Y.-L. Leu, P.-C. Kuo, B. T. M. Nguyet, D. N. Dai, T.-S. Wu, 'Isolation and synthesis of melodamide A, a new anti-inflammatory phenolic amide from the leaves of *Melodorum fruticosum*', *Planta Med.* **2013**, *79*, 288–294.

[19] C. Norbedo, G. Ferraro, J. D. Coussio, 'Flavonoids from *Achyrocline flaccida*', *Phytochemistry* **1984**, *23*, 2698–2700.

[20] H. Dong, S.-X. Chen, H.-X. Xu, S. Kadota, T. Namba, 'A new antiplatelet diarylheptanoid from *Alpinia blepharocalyx*', *J. Nat. Prod.* **1998**, *61*, 142–144.

[21] E. F. Nesmelova, I. A. Bessonova, S. Y. Yunusov, 'The structure of haplamide and the synthesis of haplobucharine', *Chem. Nat. Compd.* **1977**, *13*, 253–254.

[22] X.-N. Yang, Y.-S. Jin, P. Zhu, H.-S. Chen, 'Amides from *Uvaria microcarpa*', *Chem. Nat. Compd.* **2010**, *46*, 324–326.

[23] M. I. Choudhary, S. Maher, A. Begum, A. Abbaskhan, S. Ali, A. Khan, Shafique-ur-Rehman, Atta-ur-Rahman, 'Characterization and antiglycation activity of phenolic constituents from *Viscum album* (European Mistletoe)', *Chem. Pharm. Bull.* **2010**, *58*, 980–982.

[24] E. Wollenweber, K. Egger, 'Flavonoid-aglycones in bud excretions of *Betula ermanii*', *Z. Pflanzenphysiol.* **1971**, *65*, 427–431.

[25] R. Tanaka, S. Matsunanga, T. Sasaki, 'A new flavanone derivative from the leaves of *Tsuga diversifolia*', *Planta Med.* **1989**, *55*, 570–571.

[26] C. Pandji, C. Grimm, V. Wray, L. Witte, P. Proksch, 'Insecticidal constituents from four species of the Zingiberaceae', *Phytochemistry* **1993**, *34*, 415–419.

[27] T.-S. Wu, Y.-Y. Chan, Y.-L. Leu, 'The constituents of the root and stem of *Aristolochia cucurbitifolia* Hayata and their biological activity', *Chem. Pharm. Bull.* **2000**, *48*, 1006–1009.

[28] J. Lam, P. Wrang, 'Flavonoids and polyacetylenes in *Dahlia tenuicaulis*', *Phytochemistry* **1975**, *14*, 1621–1623.

[29] T. T. Thuy, A. Porzel, H. Ripperger, T. V. Sung, G. Adam, 'Chalcones and ecdysteroids from *Vitex leptobotrys*', *Phytochemistry* **1998**, *49*, 2603–2605.

[30] R. B. Filho, M. S. Da Silva, O. R. Gottlieb, 'Flavonoids from *Iryanthera laevis*', *Phytochemistry* **1980**, *19*, 1195–1197.

[31] G. Lindstedt, 'Constituents of pine heartwood. XXII. The isolation of pinostrobin and 3,5-dihydroxy-7-methoxyflavanone from the heartwood of *Pinus clausa* Vasey.', *Acta Chem. Scand.* **1950**, *4*, 1042–1046.

[32] S. Abbate, L. F. Burgi, E. Castiglioni, F. Lebon, G. Longhi, E. Toscano, S. Caccamese, 'Assessment of configurational and conformational properties of naringenin by vibrational circular dichroism', *Chirality* **2009**, *21*, 436–441.

[33] U. Prawat, O. Chairerk, U. Phupornprasert, A.-W. Salae, P. Tuntiwachwuttikul, 'Two new C-benzylated dihydrochalcone derivatives from the leaves of *Melodorum siamensis*', *Planta Med.* **2013**, *79*, 83–86.

[34] Q.-M. Xu, Y.-L. Liu, B.-H. Zhao, L.-Z. Xu, S.-L. Yang, S.-H. Chen, 'Amides from the stems of *Uvaria kweichowensis*', *Yaoxue Xuebao* **2007**, *42*, 405–407.
[35] A. L. Alessandri, L. P. Sousa, C. D. Lucas, A. G. Rossi, V. Pinho, M. M. Teixeira, 'Resolution of inflammation: mechanisms and opportunity for drug development', *Pharmacol. Ther.* **2013**, *139*, 189–212.
[36] M. Baggiolini, I. Clark-Lewis, 'Interleukin-8, a chemotactic and inflammatory cytokine', *FEBS Lett.* **1992**, *307*, 97–101.
[37] J. R. Bradley, 'TNF-mediated inflammatory disease', *J. Pathol.* **2008**, *214*, 149–160.
[38] C. Popa, M. G. Netea, P. L. C. M. van Riel, J. W. M. van der Meer, A. F. H. Stalenhoef, 'The role of TNF-α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk', *J. Lipid Res.* **2007**, *48*, 751–762.
[39] H. Zelová, J. Hošek, 'TNF-α signalling and inflammation: interactions between old acquaintances', *Inflammation Res.* **2013**, *62*, 641–651.
[40] J. A. Nick, N. J. Avdi, S. K. Young, L. A. Lehman, P. P. McDonald, S. C. Frasch, M. A. Billstrom, P. M. Henson, G. L. Johnson, G. S. Worthen, 'Selective activation and functional significance of p38α mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils', *J. Clin. Invest.* **1999**, *103*, 851–858.
[41] F. A. Simard, A. Cloutier, T. Ear, H. Vardhan, P. P. McDonald, 'MEK-independent ERK activation in human neutrophils and its impact on functional responses', *J. Leukocyte Biol.* **2015**, *98*, 565–573.
[42] P. G. Arndt, N. Suzuki, N. J. Avdi, K. C. Malcolm, G. S. Worthen, 'Lipopolysaccharide-induced c-Jun NH_2-terminal kinase activation in human neutrophils: role of phosphatidylinositol 3-kinase and Syk-mediated pathways', *J. Biol. Chem.* **2004**, *279*, 10883–10891.
[43] A. Etzioni, 'Adhesion molecules – their role in health and disease', *Pediatr. Res.* **1996**, *39*, 191–198.
[44] J.-J. Liu, C.-W. Song, Y. Yue, C.-G. Duan, J. Yang, T. He, Y.-Z. He, 'Quercetin inhibits LPS-induced delay in spontaneous apoptosis and activation of neutrophils', *Inflammation Res.* **2005**, *54*, 500–507.
[45] J. C. Powers, J. L. Asgian, Ö. D. Ekici, K. E. James, 'Irreversible inhibitors of serine, cysteine, and threonine proteases', *Chem. Rev.* **2002**, *102*, 4639–4750.
[46] K. B. Harikumar, A. B. Kunnumakkara, K. S. Ahn, P. Anand, S. Krishnan, S. Guha, B. B. Aggarwal, 'Modification of the cysteine residues in IκBα kinase and NF-κB (p65) by xanthohumol leads to suppression of NF-κB-regulated gene products and potentiation of apoptosis in leukemia cells', *Blood* **2009**, *113*, 2003–2013.
[47] H. Rücker, N. Al-Rifai, A. Rascle, E. Gottfried, L. Brodziak-Jarosz, C. Gerhäuser, T. P. Dick, S. Amslinger, 'Enhancing the anti-inflammatory activity of chalcones by tuning the Michael acceptor site', *Org. Biomol. Chem.* **2015**, *13*, 3040–3047.
[48] T. W. Schultz, J. W. Yarbrough, R. S. Hunter, A. O. Aptula, 'Verification of the structural alerts for Michael acceptors', *Chem. Res. Toxicol.* **2007**, *20*, 1359–1363.
[49] Y. K. Koleva, J. C. Madden, M. T. D. Cronin, 'Formation of categories from structure–activity relationships to allow read-across for risk assessment: toxicity of α,β-unsaturated carbonyl compounds', *Chem. Res. Toxicol.* **2008**, *21*, 2300–2312.
[50] A. O. Aptula, D. W. Roberts, 'Mechanistic applicability domains for nonanimal-based prediction of toxicological end points: general principles and application to reactive toxicity', *Chem. Res. Toxicol.* **2006**, *19*, 1097–1105.
[51] P. A. Jackson, J. C. Widen, D. A. Harki, K. M. Brummond, 'Covalent modifiers: a chemical perspective on the reactivity of α,β-unsaturated carbonyls with thiols via hetero-Michael addition reactions', *J. Med. Chem.* **2017**, *60*, 839–885.
[52] S. Amslinger, N. Al-Rifai, K. Winter, K. Wörmann, R. Scholz, P. Baumeister, M. Wild, 'Reactivity assessment of chalcones by a kinetic thiol assay', *Org. Biomol. Chem.* **2013**, *11*, 549–554.
[53] L. Arshad, I. Jantan, S. N. A. Bukhari, S. Jamil, 'Inhibitory effects of α,β-unsaturated carbonyl-based compounds and their pyrazoline derivatives on the phagocytosis of human neutrophils', *Med. Chem. Res.* **2018**, *27*, 1460–1471

Received June 1, 2018
Accepted August 29, 2018

