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Nigella damascena L. essential oil and its main constituents, damascenine and β-elemene modulate inflammatory response of human neutrophils *ex vivo*

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ABSTRACT

Nigella damascena L belongs to Ranunculaceae family and is mentioned in Eastern traditional medicine for the treatment of high temperatures, regulation of menstruation or catarrhal affections. The anti-inflammatory activity of compounds present in the essential oil obtained from seeds of this plant can be found in literature, however no studies on immunomodulatory activity are provided. Hence, in this work anti-inflammatory activity of *N. damascena* seed essential oil as well as damascenine and main compound β-elemene was evaluated on *ex-vivo* lipopolysaccharide (LPS)–stimulated human neutrophils. For isolation of damascenine fast and efficient protocol was elaborated using high performance countercurrent chromatography technique for the first time. Also detailed spectroscopic characteristic of damascenine was provided for the first time. Damascenine was separated from the essential oil in a mixture of petroleum ether/acetonitrile/acetone (2:1.5:0.5 v/v/v) in reversed phase mode in 12 min with 99.47% purity. Essential oil, damascenine and β-elemene presented immunomodulatory activity evaluated in LPS–stimulated neutrophils *ex vivo*. All studied samples significantly inhibited release of interleukin 1 beta (IL-1β) and interleukin 8 (IL-8). What is more, damascenine and β-elemene decreased matrix metallopeptidase 9 (MMP-9) production similar to dexamethasone. The release of tumor necrosis factor (TNF-α) was also inhibited in all range of concentrations, however the activity was weaker then activity of dexametasone. The previously reported anti-inflammatory activity of damascenine and β-elemene investigated in murine models was confirmed in our study on human neuthrophils suggesting their possible strong inhibitory effect on inflammatory response progression.

1. Introduction

Inflammation is a complex, protective and non-specific immune response to different type of harmful factors. Pathogens, irritants or damaged tissues induce inflammatory process which under normal conditions is self-limiting (Ferrero-Miliani et al., 2007). A fever, hypotension and the release of adrenocorticotrophic hormone are the first symptoms of inflammation mediated by soluble proinflammatory cytokine interleukin 1 beta (IL-1β), which is released mainly by neutrophils and monocytes (Ferrero-Miliani et al., 2007). Neutrophils infiltrate infected or damaged tissue migrating through basement membranes via action of elastase and matrix metallopeptidase 9 (MMP-9) (Delclaux et al., 1996). MMP-9 also activates IL-1β and cleavage of several chemokines (Opdenakker et al., 2001). On site, neutrophils release chemokines and cytokines (interleukin 8, IL-8; IL-1β) enhancing inflammatory response of other white blood cells, which overstimulated

Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; CID, collision-induced dissociation; COX-2, cyclooxygenase-2; D, damascenine; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FBS, fetal bovine serum; F-MLP, N-formylmethionyl-leucyl-phenylalanine; FPR, N-formylpeptide receptors; GC-MS, gas chromatography–mass spectrometry; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPCCC, high performance countercurrent chromatography; IL-8, interleukin 8; IL-10, interleukin-10; IL-1β, interleukin 1 beta; iNOS, nitric oxide synthase; LC-ESI-QTOF-MS, liquid chromatography–mass spectrometry with electrospray ionization ion source and quadrupole time of flight detector; LC-MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; MMP-9, matrix metallopeptidase 9; NMR, nuclear magnetic resonance; PAF, platelet activating factor; PBS, phosphate-buffered saline; PGE2, prostaglandin-E2; PI, propidium iodide; RP-HPLC, reversed phase high performance liquid chromatography; SOD, superoxide dismutase; TLR-4, Toll like receptor 4; TNF-α, tumor necrosis factor; EGF, epidermal growth factor; βE, β-elemene

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initiate chronic inflammation (Czerwińska et al., 2018). To prevent development of prolonged inflammation and diseases related to inflammatory conditions the use of natural substances mitigating inflammation is of high importance.

Nigella damascena L belongs to Ranunculaceae family and is mentioned in Eastern traditional medicine for the treatment of high temperatures, regulation of menstruation, catarrhal affections, as a diuretic agent and against tapeworm (Alamgir, 2017; Boniand Patri, 1977; Fico et al., 2004; Fournier, 1948). The plant produces aromatic compounds which are stored especially in the seeds and can be obtained as essential oil. Damascenine and β-elemene are major constituents of *N. damascena* L. seeds essential oil (Fico et al., 2003; Sieniawska et al., 2018; Wajs et al., 2009). Current literature on activity of essential oil is limited to antimicrobial and molluscicidal assays (Fico et al., 2004; Sieniawska et al., 2018) however damascenine was studied for analgesic, antipyretic, anti-inflammatory and antiedematous effects (Bekemeier et al., 1967; Bekemeier and Schmollack, 1967). What is more, 3-hydroxyanthranilic acid, a precursor of damascenine in plants (Robinson, 1968), induced effectively the expression of hemeoxygenase-1, an antioxidant enzyme with anti-inflammatory and cytoprotective properties (Krause et al., 2011). Also β-elemene was recently proved to attenuate the lipopolysaccharide-induced murine macrophage activation and proinflammatory factors production (Fang et al., 2018). β-elemene treatment reduced inflammation (Patra et al., 2016) and suppressed the inflammation in experimental autoimmune encephalomyelitis of optic nerve in mice models (Zhang et al., 2010). Nonetheless, the anti-inflammatory activity of *N. damascena* essential oil and its main constituents damascenie and β-elemene was not studied in human neutrophils yet.

Damascenine is a methylated derivative of 3-hydroxyantranilic acid. So far, this compound was isolated using laborious procedure described in 1912 by Ewins. This included several hours of extraction of seeds, extract treatment with 5% solution of hydrochloric acid, shaking with kerosene multiplexer, basification of aqueous layer with ammonia and additional extraction into petroleum ether (Ewins, 1912). In 2004 another time consuming method was applied. Damascenine was obtained from imbibition water from seeds. The water was extracted repeatedly with butanol and butanolic extract was chromatographed on Sephadex LH-20 yielding numerous fractions which were then combined and rechromatographed by reversed phase high performance liquid chromatography (RP-HPLC) (Fico et al., 2004). In this study, for a separation of damascenine the modern high performance counter-current chromatography (HPCCC) technique was applied. HPCCC principle utilizes the continuous liquid-liquid extraction in a solvent system composed from two immiscible phases. Compounds partition between stationary and mobile phase pumped into the column in opposite directions. This can be obtained due to centrifugal force which ensure dynamic mixing between both phases and providing good retention of a high amount of the stationary phase. HPCCC is time-saving and lossless technique, where separation lasts from several minutes to 3–4 h and substances retained in a stationary phase can be fully recovered by evaporation of the solvent (Guzleket al., 2009; Skalicka-Woźniak and Garrard, 2015).

In order to evaluate possible mechanism of anti-inflammatory activity of *N. damascena* seed essential oil as well as damascenine and main compound β-elemene on *ex-vivo* LPS–stimulated human neutrophils, fast and efficient method for isolation by HPCCC technique was performed for the first time.

2. Material and methods

2.1. Chemicals

Analytical grade acetonitrile, acetone and petroleum ether were purchased from Polish Chemical Reagents (POCH, Poland), while acetonitrile, water and formic acid for LC-MS from J.T. Baker Chemicals (Witko, Poland). Helium 5.0 with 99.999% purity was provided by (PGNiG, Poland). F-MLP (N-formylmethionyl-leucyl-phenylalanine), Hanks' balanced salt solution (HBSS), L-glutamine, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), propidium iodide (PI), 3% dextran and RPMI 1640 medium and dexamethasone (DEX) (> 95% HPLC purity) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). LPS (lipopolysaccharide) was obtained from Merck Millipore (Billerica, MA, USA). ELISA sets and BD Pharm Lyse solution were purchased from BD Biosciences (Franklin Lakes, USA), while phosphate-buffered saline (PBS) was delivered from ThermoFisher Scientific (Waltham, MA, USA).

Table 1
The partition coefficients values for damascenine in different solvent systems.

No	Solvent systems (v/v)	K
2	Petroleum ether/Acetonitrile/Acetone (7:6:1)	0.25
3	Petroleum ether/Acetonitrile/Acetone (2:1:1)[a]	0.43
4	Petroleum ether/Acetonitrile/Acetone (2:1.5:0.5)	0.62

K – partition coefficient.
[a] The solvent system becomes a single phase above 23 °C.

2.2. Studied samples

Damascenine (D) and β-elemene (βE) used in this study were isolated from *Nigella damascena* L. seeds essential oil. Commercially available seeds were purchased from Vilmorin Garden (Komorniki, Poland) and 50 g of dried and powdered seeds were subjected to 3 h hydrodistillation with 400 mL of distilled water. The essential oil was collected in a vial, dried over anhydrous sodium sulfate, and stored at 4 °C until analysis. The hydrodistillation yielded 0.5 mL of essential oil.

Essential oil was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution at the concentration of 2 mg/mL. Compounds were also dissolved in DMSO to obtain stock solutions at the concentration of 10 mM. Stock solutions were kept in −20 °C for further use. Samples were diluted in RPMI directly before experiments.

2.3. HPCCC isolation

β-elemene isolation procedure was described previously (Sieniawska et al., 2018), while damascenin isolation was developed herein for the first time using HPCCC technique. Chromatographic separation was performed on Spectrum High-performance counter-current chromatographicsystem purchased from Dynamic Extraction Co., Ltd. (Slough, Berkshire, UK). In order to select the most suitable two-phase solvent system the partition coefficient (K) values were calculated for damascenine in a different solvent combinations according to the protocol reported previously (Sieniawska et al., 2016). Finally, the separation was carried out using petroleum ether, acetonitrile and acetone in a ratio of 2:1.5:0.5 (v/v) as a solvent system, in a reversed phase mode at 26 °C. Preparative scale was applied with 1600 RPM for bobbins rotation and 6 mL/min flow rate in 137 mL coil. Two-phase solvent system and sample preparation were described in details previously (Sieniawska et al., 2016). The eluate was monitored at 210 nm, then 1-min fractions were analyzed by GC-MS. Identification of isolated damascenine was additionally confirmed by LC-ESI-QTOF-MS and NMR.

2.4. GC-MS analysis

Shimadzu GC-2010 Plus coupled to a Shimadzu QP2010 Ultra mass spectrometer (Japan) with a fused-silica capillary column ZB-5 MS (30 m, 0.25 mm i.d., 0.25 mm film thickness; Phenomenex, USA) was used for evaluation of obtained essential oil and for fractions monitoring. The oven temperature was set at 50 °C and held for 3 min, then

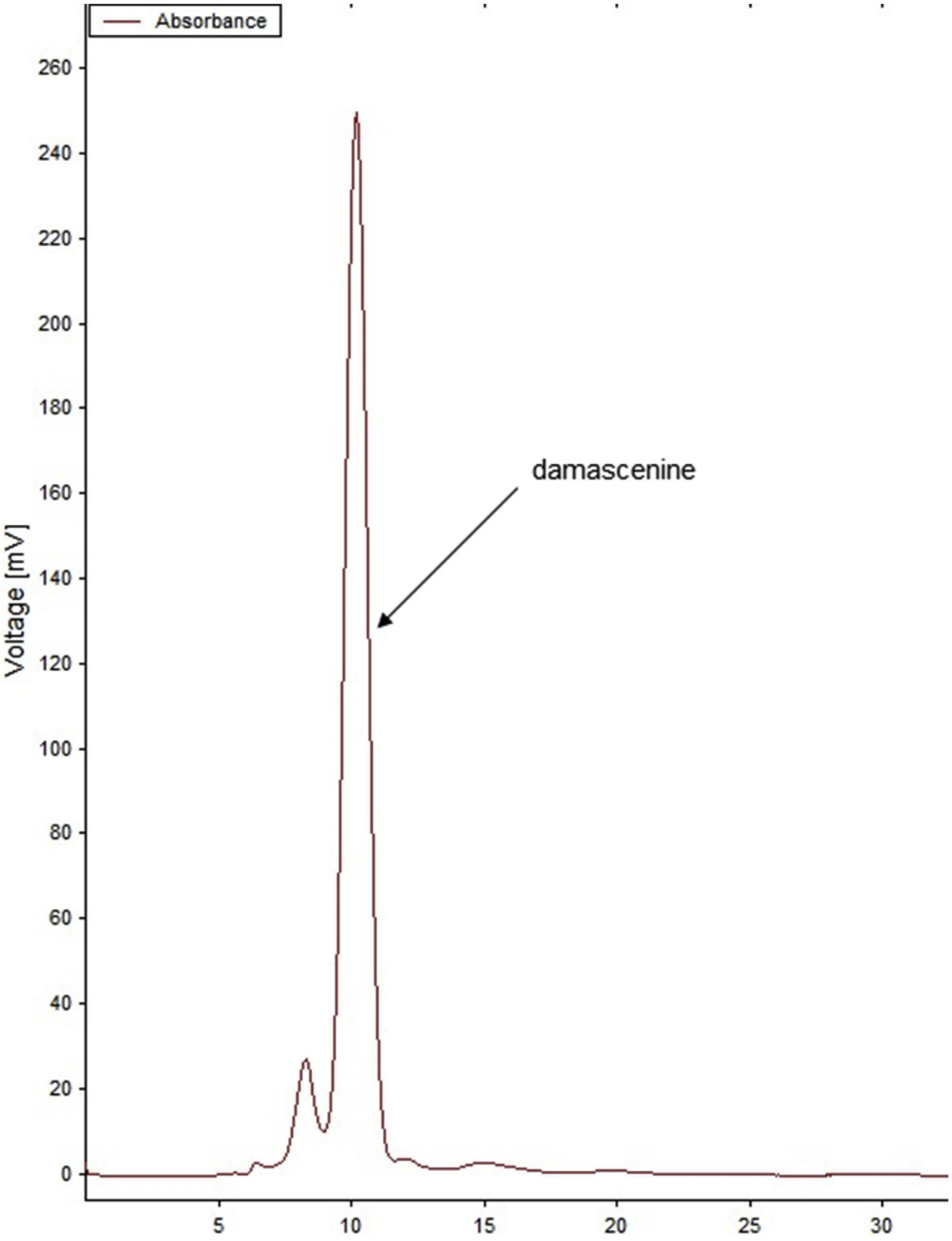

Fig. 1. HPCCC chromatogram of *N. damascena* seeds essential oil separated in a mixture of petroleum ether/acetonitrile/acetone (2:1.5:0.5) in reversed phase mode.

Table 2
The results of separation of 200 mg of *N. damascena* essential oil in a mixture of petroleum ether/acetonitrile/acetone in the ratio 2:1.5:0.5 – solvent system 3, in a reversed phase mode.

Fraction (min)	Purity (%)	Yield (mg)	Efficiency (%)
9.5	98.0	4.6	17
10.5	99.5	13.5	54
	Total	18.1	71

was rised to 250 °C at a rate of 8 °C/min and this temperature was held for 2 min. The temperature of the injector, interface, and ion source were kept at 250, 250, and 220 °C respectively. Helium was used as carrier gas with a flow rate of 1 mL/min. Split ratio was set at 1:20. Electron impact ionization with 70 eV was performed. Mass spectral data were acquired in the scan mode in the m/z range 40–500 with the scan rate 0.20 s per scan (Sieniawska et al., 2016). Homologous series of *n*-alkanes (C_8—C_{24}) was used to determine retention indices under the same operating conditions. Identification was performed by comparison of mass spectra of compounds and their retention indices with

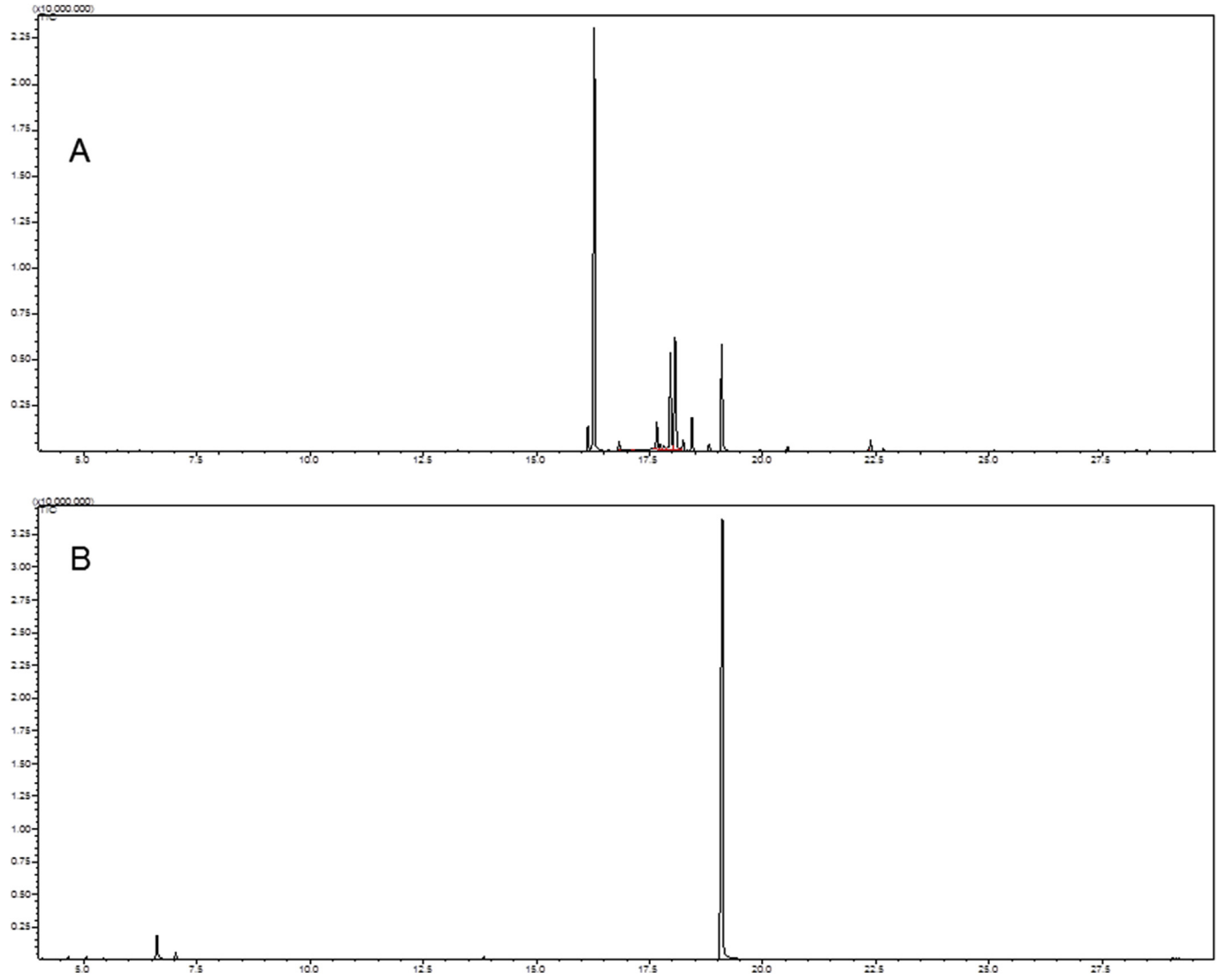

Fig. 2. GC chromatograms of *N. damascena* essential oil (A) and isolated damascenine (B).

computer-supported spectral libraries (Mass Finder 2.1 and NIST database).

2.5. LC-ESI-QTOF-MS analysis

Agilent 1200 Infinity HPLC chromatograph hyphenated with QTOF 6530B Accurate-Mass QTOF LC/MS system equipped with Dual Agilent Jet Stream spray source (ESI) (Agilent Technologies, Santa Clara, CA, USA) connected with N2 generator (Parker Hannifin Corporation, Haverhill, MA; generating N2 at purities > 99%) was used for LC-ESI-QTOF-MS analysis. The HPLC was carried out in 25 °C on Gemini® C18 column with TMS endcapping (3 μm i.d., 110 Å, 100 × 2 mm) and SecurityGuard™ column. Mobile phase was composed of: 95% ACN in water (A) and 95% ACN in water (B), both phases with addition of 0.2% formic acid. The following gradient was applied: 0–10 min 0% of B; 10–15 min from 0 to 8% of B; 15–22 min 8% of B. The flow rate was 0.250 mL/min and injection value: 10 μl. ESI-QTOF-MS analysis was performed in positive ionization mode, with fragmentor energy of 150 V; drying gas temp: 300 °C, drying gas flow: 12 l/min, sheath gas temp: 350 °C, sheath gas flow: 12 l/min; nebulizer pressure: 35 psig, capillary V (+): 4000 V, skimmer 65 V. Acquisition parameters for MS and Auto MS/MS mode were in a mass range 10–1000 amu for 1 spectra/s acquisition. The collision-induced dissociation (CID) energy was optimized in the range 10–40 V.

2.6. NMR analysis

1D (1H-NMR, 13C-NMR) and 2D (NOESY, HMBC, HSQC) spectra were recorded at 25 °C on Bruker NMR Ascend 600 spectrometer (Bruker Corporation, Germany) operating at a proton and carbon NMR frequency of 600.11 MHz. All the spectra were recorded in deuterated methanol (CD_3OD) using the same solvent for internal lock at relaxation time 4.07 s.

2.7. Isolation of neutrophils from buffy coats

The buffy coat was prepared from peripheral venous blood collected from healthy human donors (male, < 35 years old) at the Warsaw Blood Donation Centre. Donors were confirmed to be healthy. Neutrophils were isolated by performing a density gradient centrifugation (Pancol, Pan-Biotech) followed by a dextran separation (3% Dextran). Remaining red blood cells were removed using a lysis solution (Czerwińska et al., 2018). The purity of neutrophils preparation was over 97%. After isolation, cells were suspended in (Ca^2+)- free HBSS, (Ca^2+)-free PBS or RPMI 1640 culture medium and maintained at 4 °C before use.

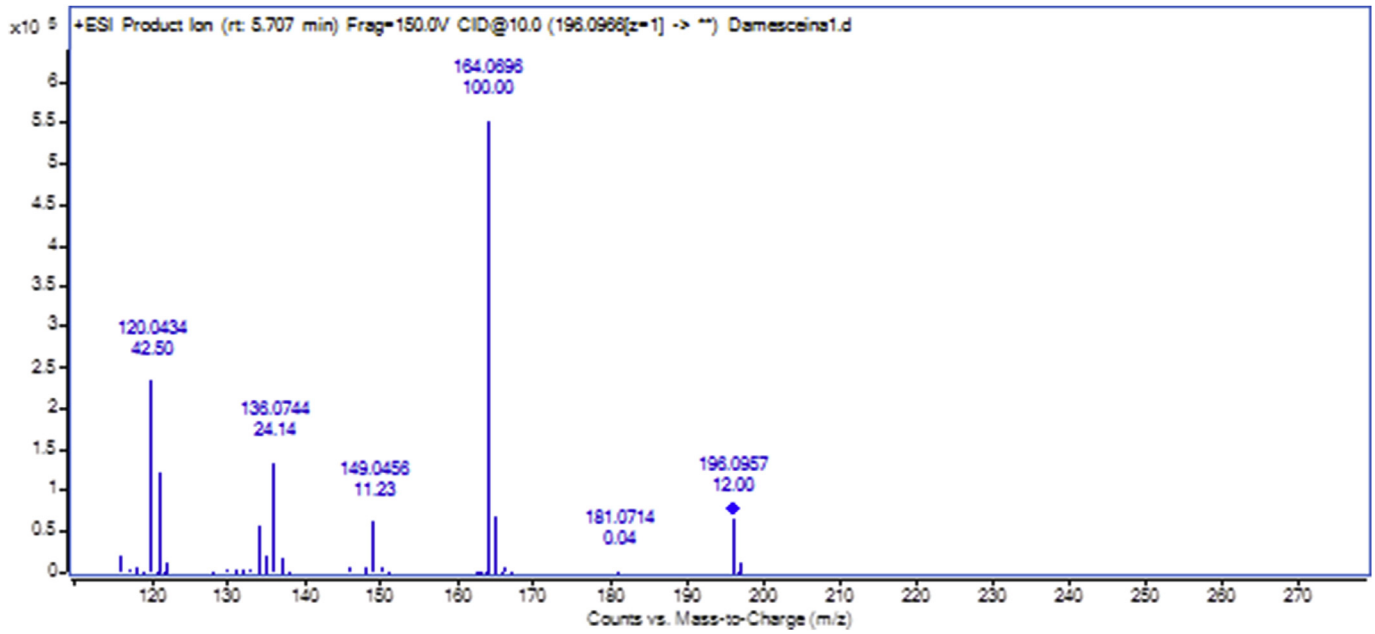

Fig. 3. ESI(+)-QTOF-MS spectrum of damascenine.

2.8. Evaluation of viability, IL-8, IL-1b, MMP-9 and TNF-α production

Neutrophils (2.0×10^6/mL) were cultured in 96-deep well plates in RPMI 1640 medium with 10% FBS, 10 mM HEPES, and 2 mM l-glutamine in the absence or presence of LPS (100 ng/mL) for 24 h at 37 °C with 5% CO_2 in the absence or presence of test compounds (25, 50 and 75 μM for pure compounds and 6.125, 12.5 and 25 μg/mL for essential oil) added to 1 mL of cell suspension 1 h before stimulation. After 24 h plates were centrifuged (2000 RPM; 10 min; 4 °C) and supernatants were collected. The production of cytokines by stimulated neutrophils was evaluated by ELISA tests following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA or R&D Systems, Minneapolis, MN, USA). Dexamethasone (25, 50 and 75 μM) was used as a positive control. Neutrophils' viability was determined by staining with propidium iodide (PI). In short, 400 μl of cold binding buffer containing IP was added and the cells were analyzed by flow cytometry (FACSCalibur) within 1 h after labelling and data from 10 000 events were recorded.

2.9. Statistical analysis

The results were expressed as a mean ± SD of the indicated number of experiments. Statistical significance of differences between the mean values were calculated using one-way analysis of variance (ANOVA) with Tukey's post hoc test, Dunnet's post hoc test or non-parametric Kruskal-Wallis test. All analyses were performed using STATISTICA software v. 10.0 PL (StatSoft, Poland). Statistical significance was set at $*p < 0.05$.

3. Results

3.1. Isolation and identification of damascenine

As the best separation of the compound can be obtained when partition coefficient value (K_{val}) is in a range between 0.5 and 2.0 (Skalicka-Wozniak and Walasek, 2014), series of solvent systems were tested. The satisfactory K_{val} was noticed when the mixture of petroleum ether/acetonitrile/acetone in the ratio 2:1.5:0.5 was applied (Table 1). Lower K_{vals} were determined for solvent systems 1 and 2, however solvent system 2 was not stable in the temperature above 23 °C and become a single phase. Due to quite low K_{val} the separation was fast, however demascenine eluted in solvent system 1 was of lower purity (max 96%) compared to solvent system 3 (max 99.6%). Therefore the final isolation was performed in the solvent system 3 which had also good retention of stationary phase (78%) and enabled the elution of damascenine in less than 12 min (Fig. 1). Less polar compounds were retained in a stationary phase. The sample load insignificantly influenced purity of separated compound (200 mg sample – 99.47% purity; 165 mg sample – 99.6%). 17 mg of damascenine was obtained from 200 mg of essential oil with 71% efficiency in a single run (Table 2).

Chromatograms shown on Fig. 2 confirmed successful isolation of damascenine which identity was further proved by LC-ESI-QTOF-MS and NMR. Isolated compound produced a molecular ion at m/z 196 $[M+H]^+$ (exact mass 196.0974 Da) and daughter ions at m/z 181, 164, 149, 136, 120 (Fig. 3). Fragment ion at m/z 181 was an unstable radical $[M + H- 15]^{+\bullet}$ with abundance less than 0.05%, resulting from loss of methyl group (Fig. 4). The main fragmentation pathway went through ion at m/z 164 $[M + H– 15–17]^+$ being a consequence of additional loss of methoxy group and hydrogen atom from amine group and resulting in closing the ring. The subsequent loss of methyl group yielded radical cation at m/z 149 [M + H– 15-17-15] which was finally transformed into ion at m/z 120 [M + H– 15-17-15-29].

^{1}H NMR spectrum revealed the presence of 3 Me groups and 3 aromatic ring protons typical for ABC spin system at C4,5,6 positions. Three Me singlets were respectively at 2.86 ppm (HN−CH_3), 3.73 ppm (OC−$\underline{OCH_3}$) and 3.75 ppm (OCH_3), whereas H4, H5 and H6 protons were respectively at: 6.95 ppm (1H, dd, J = 7.93 and 1.49 Hz), 6.63 ppm (1H, t, J = 8.01 Hz) and 7.37 ppm (1H, dd, J = 8.09 and 1.49 Hz). NH signal has not been recorded in deuterated methanol. It was clear that the compound possess the same constituents as damascenine but whether it is that compound or its isomer further NMR including 2D NMR analysis was needed. In ^{13}C NMR spectrum 10 carbon signals have been identified. They are as follows: 169.0 ppm (C=O), 150.8 ppm (C3), 143.9 ppm (C2), 122.9 ppm (C6), 117.4 ppm (C5), 116.0 ppm (C4), 115.0 ppm (C1), 55.1 ppm (OCH_3), 50.8 ppm (OC−$\underline{OCH_3}$) and 33.0 ppm (NH−CH_3). Proper values of ^{13}C signals attached to the proton ones were established using also HSQC spectra. HMBC and NOESY spectra were also used as confirmation of damascenine as localization of its constituents was exactly the same. In Fig. 5 correlation cross-peaks observed in those 2D NMR spectra has been presented.

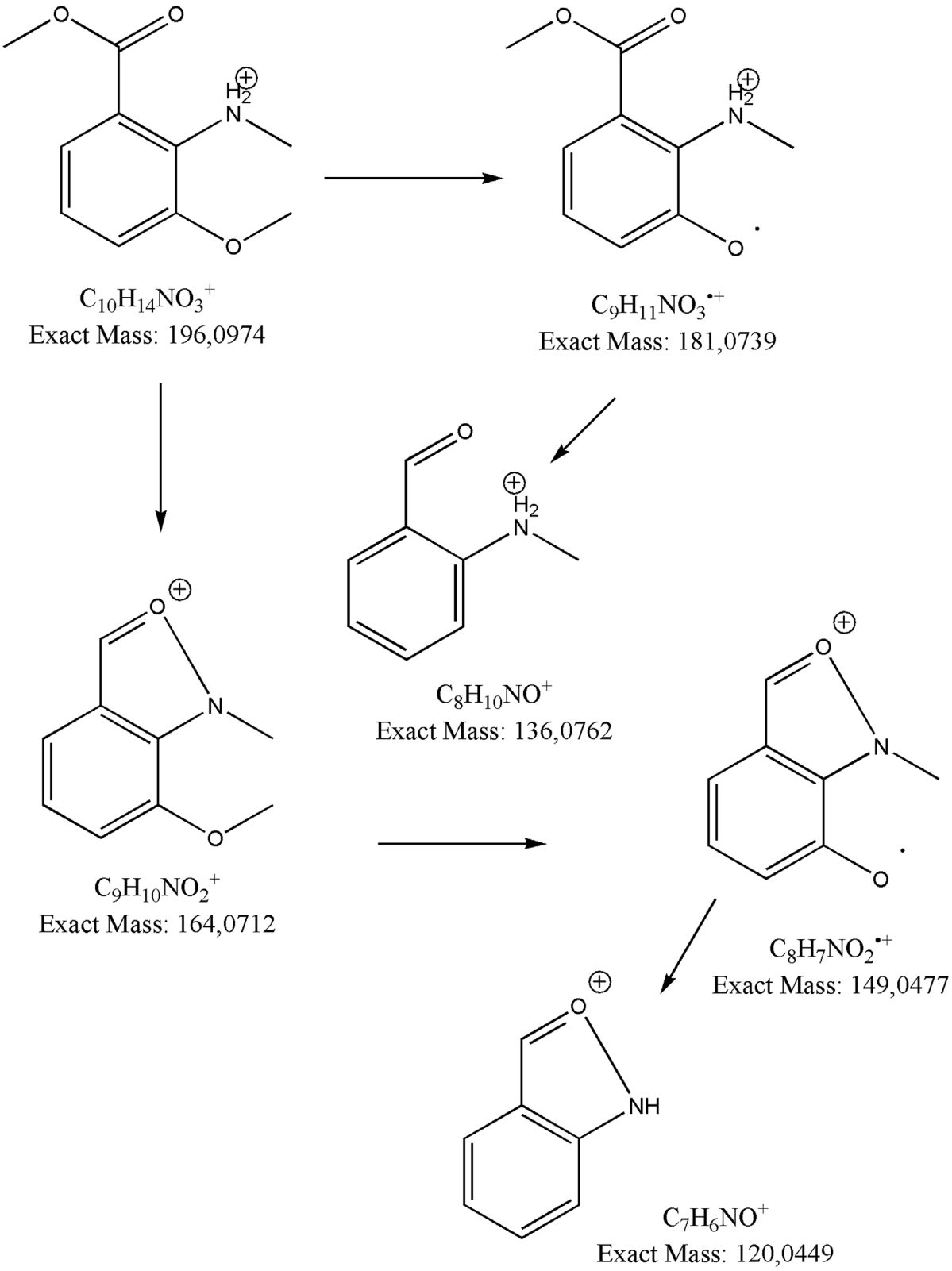

Fig. 4. Fragmentation pathways of damascenine.

3.2. Bioactivity of the essential oil and isolated compounds

During the viability assay it was confirmed that neither EO nor both investigated compounds were cytotoxic at whole concentration range used in the assays (data not shown).

The potential anti-inflammatory activity of EO was investigated in human neutrophils *ex vivo* model. The influence on the production of pro-inflammatory cytokines (IL-8, IL-1β and TNF-α) and enzyme, matrix metalloproteinase-9 (MMP-9) after stimulation with LPS was evaluated. First the activity of EO was tested. The EO was active towards all three cytokines at the highest used concentration (25 μg/mL). The most promising anti-inflammatory activity of the EO was observed

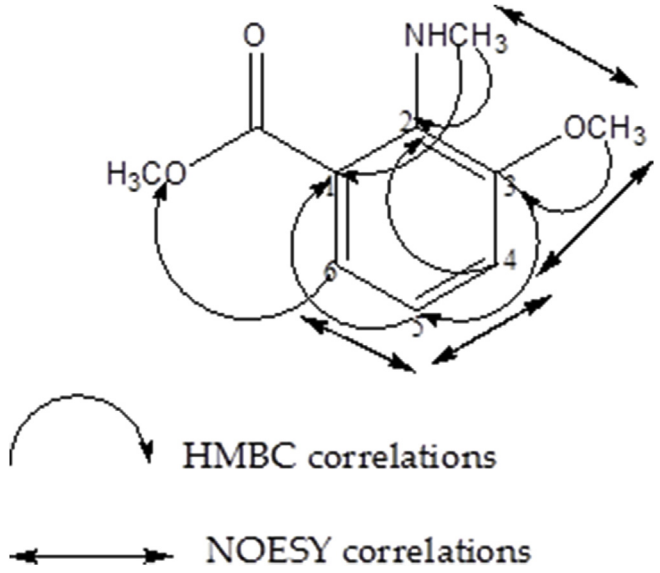

Fig. 5. Selected HMBC and NOESY correlations of damascenine.

in the case of influence on the production of IL-1β (Fig. 6). The EO was able to decrease the production of this cytokine in a concentration dependent manner starting from 6.125 μg/mL. Only concentration of 25 μg/mL of EO was able to decrease the production MMP-9.

In order to determine which constituents of the EO might contribute to the observed anti-inflammatory potential, two major compounds - β-elemene and damasceine was evaluated in the same manner. Both compounds were able to significantly decrease the production of IL-8 only in the concentration of 75 μM (Fig. 7) and activity was weaker than that observed for the positive control – dexamenthasone. In IL-1β and TNF-α assays both compounds were active at all used concentration levels (Fig. 7) and activity of compounds was similar in both cases. However, in TNF-α experiments constituents were weaker inhibitors than positive control, while in IL-1β assays they acted with similar strength (no statistically significant differences were observed). In the case of the influence on MMP-9 production β-elemene and damasceine were active only at two highest concentrations used and the observed activity was similar for both compounds (Fig. 7) as well as for dexamethasone used as a positive control.

4. Discussion

The fast, selective and effective separation of natural compounds is of high importance for their availability for biological testing. The information about biological activity of damascenine is very poor, most probably because of lack of method for its efficient separation. Hence, in this study, we elaborated the effective protocol for fast isolation of this compound. The HPCCC technique was proved to be the efficient and simple, one step separation of damascenine. 71% of damascenine present in the sample loaded into the column was recovered and 54% of this amount with purity over 99% (Table 2). The previous methodology described by Ewins gave good purity of isolated compound (95%), however the yield was very low 0.15% (Ewins, 1912). Also, classical separation involving liquid-liquid extraction, column chromatography on Sephadex and preparative HPLC (Fico et al., 2004) resulted in isolation of lower amount of targeted compound comparing to our study (20 mg from 430 mg of sample and 17 mg from 200 mg sample, respectively). In the second method the purity of obtained damascenine was not provided.

HPCCC technique was previously used for successful preparative isolation of varied alkaloids (Skalicka-Woźniak and Garrard, 2015), however the separation of protoalkaloids is less described. Only cathine and norephadrine from *Catha edulis* were obtained by means of HPCCC (Atlabachew et al., 2016). Alkaloids were eluted using solvent system of hexane/ethyl acetate/methanol:/2% aqueous NH_4OH (1:4:1:4) in 90 min run. However the sample preparation required several steps of acid-base extraction and preparation of oxalate salts (Atlabachew et al., 2016). As can be seen, the matrix strongly influences the protoalkaloids separation process. However, applying properly selected solvent system and due to different polarity of targeted compound and other compounds present in the *N. damascena* essential oil, HPCCC technique enabled selective isolation of damascenine in the short time. Being among the main constituents of the essential oil damascenine was

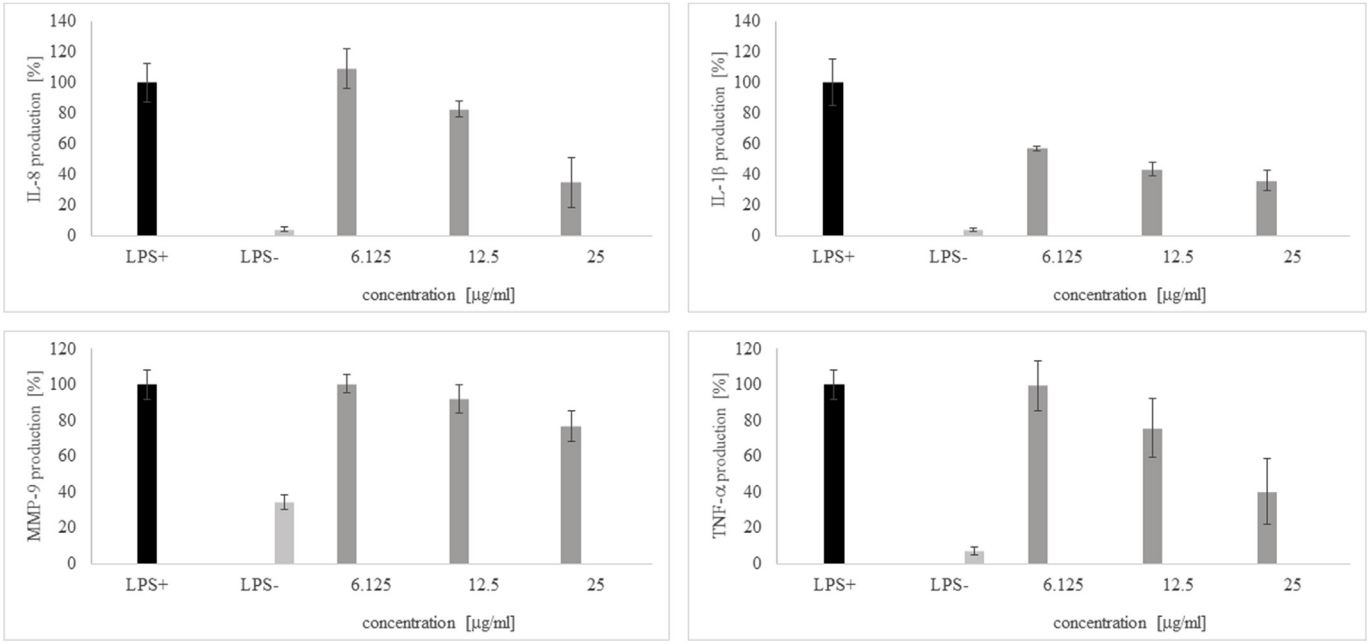

Fig. 6. Effect of *Nigella damascena* essential oil on cytokines and MMP-9 production from LPS-stimulated (100 ng/mL) neutrophils. Data were expressed as mean ± SEM of three separate experiments performed with cells isolated from independent donors assayed in triplicate. *$p < 0.05$ – statistically significant versus stimulated control (Dunnett's post hoc test); #$p < 0.001$ a statistically significant versus non-stimulated control.

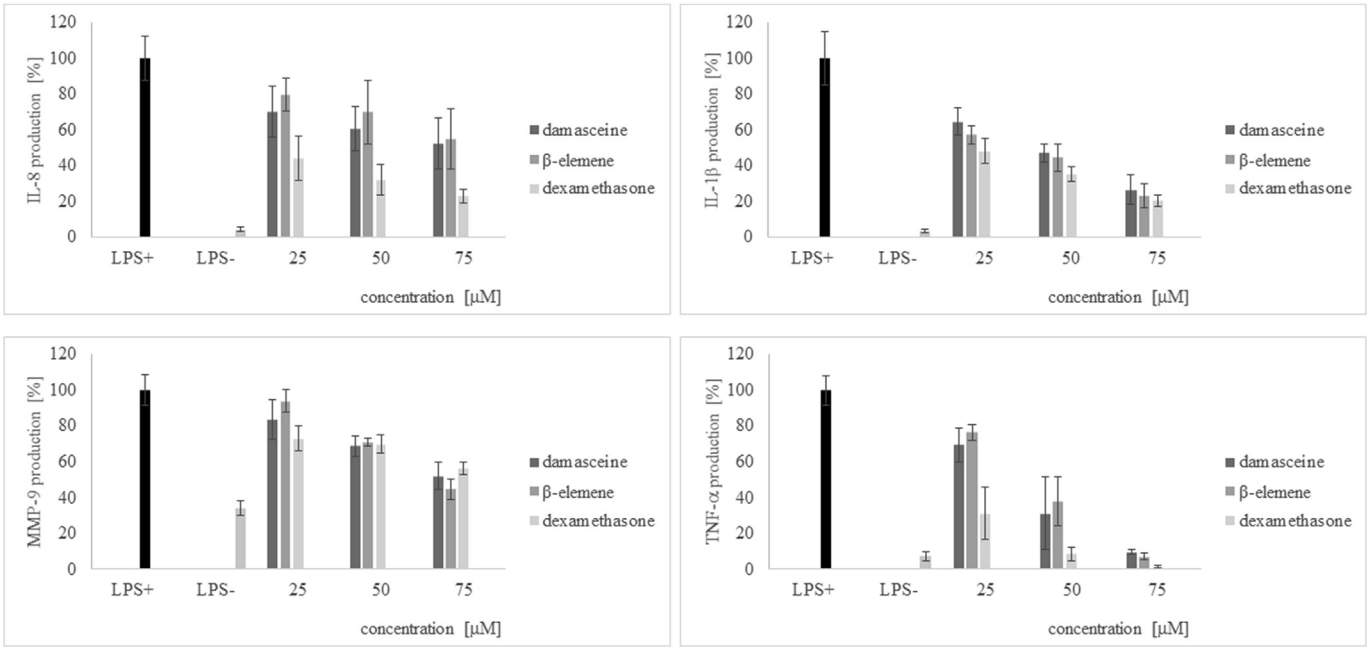

Fig. 7. Effect of damascenine, β-elemene and dexamethasone on cytokines and MMP-9 production from LPS-stimulated (100 ng/mL) neutrophils. Data were expressed as mean ± SEM of three separate experiments performed with cells isolated from independent donors assayed in triplicate. *$p < 0.05$ – statistically significant versus stimulated control (Dunnett's post hoc test); #$p < 0.001$ a statistically significant versus non-stimulated control.

obtained with a good yield (17 mg per 50 g of seeds). Additionally, full NMR spectra set including 2D NMR ones has been recorded for the first time. Also, HR MS fragmentation pathways has been proposed for this compound.

Isolated damascenine, β-elemene and essential oil presented immunomodulatory activity evaluated in LPS–stimulated neutrophils *ex vivo*. All studied samples significantly inhibited release of IL-1β, which being a potent pro-inflammatory cytokine is essential for host-defence responses to infection and injury (Dinarello, 1996). Inflammatory symptoms induced by IL-1β are mediated by cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS) which overstimulated lead to chronic inflammation and acute tissue injuries (Dinarello, 2010). Damascenine and β-elemene inhibited release of IL-1β with similar strength as dexamethasone. EO was not so potent, however it was able to decrease the production of this cytokine in a concentration dependent manner. The results suggest potential of studied samples in prevention of neutrophils inflammatory response progression. Isolated compounds and EO were able to significantly decrease the production of IL-8 by LPS–stimulated neutrophils only in the highest concentration and observed activity was lower than dexametasone. Hence their usefulness in suppression of effects induced by IL-8, like physiological inflammation induced mainly by infection with microbial products or pathological severe inflammatory reactions leading to diseases like Alzhimer's diseases (Sutinen et al., 2012) may be considered only as supplementary. Although human neutrophils produce TNFα in quite low amounts, it amplifies release of neutrophil-derived cytokines and chemokines (Tecchio et al., 2014) and plays a crucial role in the promotion of their adhesion to endothelium which is needed in tissue infiltration by neutrophils (Gabrilovich, 1999; Tecchio et al., 2014). All investigated samples inhibited the release of this factor from stimulated neutrophils. Isolated compounds showed similar activity in all range of concentrations, however the activity was weaker then activity of dexametasone. Nethertheless studied essential oil and its main constituents may attenuate the effects evoked by TNFα release from neutrophils and may influence of development of inflammation. In the case of the influence on MMP-9 production, damascenine and β-elemene revealed activity similar to dexamethasone. This indicates that they could protect tissues from proteolytic activity of released by neuthrophils matrix degradation enzyme and subsequently they could inhibit neuthrophils migration. Such activity would be useful in limitation of inflammatory process.

The anti-inflammatory effects exerted by natural products can be mediated via different concomitant mechanisms. One of them may depend on the ability to scavenge reactive oxygen species (ROS), which produced mainly by macrophages and neutrophils induce neutrophils recruitment at the inflamed tissues (Fialkow et al., 2007). Stimulation of expression/release of antioxidant enzymes may lead to inhibitory effects on the production of pro-inflammatory mediators (Locatelli et al., 2017). Such mechanism was observed in case of 3-hydroxyanthranilic acid (3-HAA), a precursor of damascenine in plants (Robinson, 1968). 3-HAA was proven to induce effectively the expression of hemeoxygenase-1 (antioxidant enzyme with anti-inflammatory and cytoprotective properties) in astrocytes and in mouse macrophages (Krause et al., 2011; Oh et al., 2004). Also β-elemene showed slight effect on the expression and activity of hemeoxygenase-1. Additionally, it presented ability to increase of superoxide dismutase (SOD) activation resulting in suppression of the overproduction of ROS in human umbilical vein endothelial cells (Liu et al., 2015). β-elemene blocked H_2O_2-induced monocyte-endothelial cells interactions (evaluated *in vitro*) leading to chronic inflammation and atherosclerosis (Liu et al., 2015). The indirectly related to the inhibition of ROS formation is the inhibition of functional N-formylpeptide receptors (FPR) expressed in high levels in neutrophils. The finding FPR antagonists could be beneficial as neutrophils activated via FPR begin chemotactic locomotion and are activated at sites of infection. It usually generates ROS, mainly through the conversion of molecular oxygen to superoxide anion and stimulates other proinflammatory factors (Mollica et al., 2012a, 2012b).

The other possible mechanism via which tested substances could show immunomodulatory activity is the regulation of cyclooxygenase-2 (COX-2)-dependent prostaglandin-E2 (PGE2) pathway. An inducible early response gene COX-2 may be activated by LPS, IL-1β, TNF-α, epidermal growth factor (EGF) or platelet activating factor (PAF). The up-regulation and over-expression of COX-2 is mainly associated with inflammation because it leads to over-production of PGE2 which in turn up-regulate several signaling pathways and down-regulate apoptotic

proteins (Gandhi et al., 2017). The inhibitory activity (over 70%) against COX-2 enzyme was observed *in vitro* for *Nigella damascena* n-hexane seeds extract at 100 μg/mL (Landa et al., 2009) showing this mechanism could be possible also in case of essential oil, which contain similarly non-polar compounds. What is more, it was observed that β-elemene downregulated the expression of nitric oxide synthase (iNOS), COX-2, TNF-α, interleukin-6 (IL-6), interleukin-10 (IL-10) and IL-1β, and their upstream signaling molecules: Toll like receptor 4 (TLR-4) and myeloid differentiation primary response protein (MyD88) suggesting that anti-inflammatory activity of β-elemene is caused by suppression of TLR4-mediated proinflammatory signaling cascades and through the regulation of COX-2-dependent prostaglandin-E2 (PGE2) pathway in the studied LPS-mediated inflammatory response (Fang et al., 2018; Patra et al., 2016). Furthermore, our work indicates that inhibition of MMP-9 observed for damascenine and β-elemene could protect tissues from proteolytic activity of released by neuthrophils matrix degradation enzyme and could inhibit neuthrophils migration.

The data presented in this work support the previous findings related mainly to anti-inflammatory activity of β-elemene, however it also shows the ability of *N. damascena* EO and damascenine to inhibit the release of proinflammatory cytokines (IL-1β, IL-8, TNF-α and MMP9) from human neutrophils. Damascenine was previously studied for its analgesic, antipyretic, anti-inflammatory and antiedematous effects in animals (Bekemeier et al., 1967; Bekemeier and Schmollack, 1967), however the mechanism of these activities was not explained. Our work is a first step for deeper understanding of anti-inflammatory activity of studied samples, nevertheless mechanisms by which *N. damascena* EO, damascenine and β-elemene showed immunomodulatory activity are very likely multifactorial.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.057.

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