Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *81* (1) 1–12 (2016)
JSCS–4821

UDC **Sclerochloa dura*:547.295.96:547.426.1+547.918:615.276–188
Original scientific paper

A novel compound isolated from *Sclerochloa dura* has anti-inflammatory effects

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(Received 19 January, revised 26 June, accepted 28 June 2015)

Abstract: The activation of phospholipase A2 (PLA_2) by means of pro-inflammatory cytokines results in the subsequent release of arachidonic acid (AA) and generates eicosanoids, which further propagate inflammation. Through cyclooxygenases (COX1/2) responsible for the enzymatic conversion of AA to eicosanoids, non-steroidal anti-inflammatory drugs are effective in relieving the pain and discomfort of inflammation. Using the AA release assay as a guide for biological and anti-inflammatory activity, the novel compound 1-*O*-(3-*O*-linolenoyl-6-deoxy-6-sulfo-*α*-D-glucopyranosyl)glycerol (**1**) together with five known compounds, *i.e*., isovitexin, byzantionoside B, tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl) ether 7-*O*-*β*-glucopyranoside, 1-*O*-feruloylglycerol and tricin 7-glucoside, isolated from the methanol extract of the aerial parts of *Sclerochloa dura* using LC techniques (Sephadex LH-20 column chromatography, preparative HPLC and semi-preparative HPLC), were evaluated. All the isolated compounds were identified using spectroscopic methods, *i.e*., NMR spectroscopy and MS spectrometry. The novel compound **1** was found to be an effective inhibitor of AA release with an IC_{50} value of 0.09±0.03 mg mL^{-1}.

Keywords: arachidonic acid; anti-inflammatory activity; phospholipase A2 inhibitor; Sephadex LH-20; HPLC; Cell line SW982.

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doi: 10.2298/JSC150119055B

INTRODUCTION

It is generally known that plants produce many chemical compounds, such as alkaloids, flavonoids, proteins, amino acids, bioactive peptides, sugars, biopesticides and others. Plants have always been the centre of attention in the history of traditional medicine, because they produce vital and complex molecules, which can be used either directly or in modified form to cure or alleviate a number of diseases. For example, berberine (alkaloid) is well known for its anti-inflammatory activity.[1] It is present in a number of plants, such as *Hydrastis canadenis*, *Berberis vulgaris*, *B. aristata*[2] and *Rhizoma coptidis*.[3] Similarly, quercetin is an anti-inflammatory compound that belongs to the class of flavonoids.[4] It can be isolated from many plants, for instance *Helminthostachys zeylanica*.[5] Bromelain (a mixture of sulfur-containing proteases) obtained from the stem of *Ananas comosus* also exhibits anti-inflammatory activity.[6] Some other classes of compounds showing anti-inflammatory activity are terpenoids, phenols and polyphenols.[7]

There are many species of plants used as traditional medicines that contain potential candidate compounds for the development of anti-inflammatory drugs.[6,8] *Sclerochloa dura* (L.) P. Beauv. may be an example of a plant with beneficial medicinal properties; however, its traditional use is not well documented. This plant, known also as common hardgrass or fairground grass, belongs to family Poaceae Barnhart, which involves more than 700 genera and almost 50,000 species. It is an annual plant, with flat leaves and a procumbent or erect steam up to 16 cm in length.[9] The inflorescence is a crowded, one-sided series of flattened spikelets. The plant is a common inhabitant of areas with heavy traffic, *e.g.* along dirty roads, on play yards and pathways. It is widely spread in the moderate climate zone of almost all continents on the North Hemisphere and was also introduced in Australia.[10] This plant has been used traditionally in small communities in South–East Serbia for the treatment of menstrual disorders characterized by excessive bleeding and pain. Professor Aleksandar Igić (Medical Faculty, University of Niš, Serbia) in a personal correspondence reported that in this region tea or decocts of *S. dura* have been used by women having menstrual disorders; according to the statements of subjects, the oral intake of a boiled water extract of the plant results in pain relief and significantly reduces bleeding.[9] In a quest to identify the chemical composition and biological properties of *S. dura*, its crude extract and subsequent fractions were tested on human fibroblastlike synoviocyte cell line SW982 (stimulated with interleukin-1β) to determine its effects on arachidonic acid (AA) release. It was recently shown that the *S. dura* extract contains one or several compounds with the ability to modulate the activity of phospholipase A_2 (PLA_2) enzymes.[11]

The PLA_2 enzymes hydrolyze fatty acids, such as AA, from the *sn*-2 ester bond of membrane glycerophospholipids. The ω-6 fatty acid (AA) is a substrate

for eicosanoids such as prostaglandins and leukotrienes, which are potent lipid mediators of inflammation.[12] The PLA_2 enzymes are grouped into five main categories, namely, cytosolic PLA_2s ($cPLA_2$), secretory PLA_2s ($sPLA_2$), Ca^{2+}-independent PLA_2s ($iPLA_2$), platelet-activating factor acetylhydrolases (PAF-AH), and lysosomal PLA_2s.[13] Apart from their role in normal physiological functions, the different isotypes are implicated in a variety of diseases[14] and ever since the $cPLA_2$ inhibitor arachidonyl trifluoromethyl ketone (ATK, AACOCF3) was introduced in 1993,[15] there has been great interest in the development of inhibitors that target specific PLA_2 subtypes.[16,17]

Most chronic non-communicable diseases, such as atherosclerosis, rheumatoid arthritis, diabetes and chronic respiratory diseases, are characterized by a considerable inflammatory component. These are the leading cause of global death. Inflammation is an acute response to harmful stimuli that normally abates after the challenge is cleared. In chronic inflammatory diseases, the response is not attenuated and the inflammation in the tissue is not completely resolved. Instead, prolonged signaling ensures a persistent inflammatory environment. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β), are abundant and activate myriad of intracellular effector enzymes, including PLA_2 enzymes. The activation of PLA_2 enzymes results in the subsequent release of AA and the generation of eicosanoids, creating a self-perpetuating process.[18–20] Several studies using knockout mice or $cPLA_2$-IVa inhibitors have highlighted the potential for targeting this enzyme in order to quench inflammation and ameliorate disease.[21]

The presence of compounds in the extracts of *S. dura* that inhibit AA release and may, hence, exert overall anti-inflammatory properties[11] is in accordance to its traditional use to relieve discomfort associated with menstrual disorders. Indeed, increased availability of AA was associated with heavy menstrual bleeding.[22] An elevation of the levels of prostaglandins, such as PGE_2 and PGF_2, in the endometrium of women with heavy menstrual bleeding compared to women with normal menses was documented.[23,24] Prostaglandins play an important role in uterus contractions[25] and are considered as major factors in primary dysmenorrhea. Non-steroidal anti-inflammatory drugs target the cyclooxygenases (COX1/2) responsible for the enzymatic conversion of AA to eicosanoids, and can therefore effectively relieve pain associated with dysmenorrheal.[24]

The present study covers the process of subfractionating the methanolic extract of *S. dura*, which lead to the isolation, as well as identification, of the novel compound 1-*O*-(3-*O*-linolenoyl-6-deoxy-6-sulfo-α-D-glucopyranosyl)glycerol (**1**) along with five known compounds, which have not hitherto been reported to show anti-inflammatory effects by affecting the PLA_2 enzyme activity (Fig. 1). All of the identified compounds expressed anti-inflammatory activity by showing modulation of the PLA_2 activity in an *in vitro* AA release assay.

EXPERIMENTAL

General

For extraction, a VWR ultrasound cleaner (USA), a Büchi rotavapor R-200 (UK), a Büchi rotavapor RII (UK) and an isomantle from Isopadisomantle (UK) were used. The medium pressure liquid chromatography (MPLC) system used was from Kronlab GmbH with a Prepcon 4.47 data system. The stationary phase used for the MPLC was Polygoprep 60–50 RP-18 (Macherey & Nagel). Water from a Millipore Elix 5 system, methanol from Fisher Scientific (UK) and 2-propanol from VWR (USA) were used for the mobile phase. A SEPBOX system (Fa. SEPIAtec) was used for the preparative and semi-preparative HPLC. The mobile phase components were water obtained from a Millipore Elix 5 system, methanol from Fisher Scientific (UK) and acetonitrile from VWR (USA). Detection was realized using ELSD (Sedex 75) and UV (Merck, λ = 250 nm) detectors. For preparative HPLC, a Kromasil C18 column (250 mm×50 mm, 10 μm) was used. For the semi-preparative HPLC, a Kromasil C18 column (250 mm×16 mm, 10 μm) and a Nucleodur C18 Isis column (100 mm×10 mm, 5 μm) were used. The human fibroblastlike synoviocyte cell line SW982 was purchased from ATCC (UK). Recombinant human IL-1β was purchased from Roche (UK); arachidonyl trifluoromethyl ketone (ATK, $AACOCF_3$) was from Enzo Life Sciences, phosphate-buffered saline solution (PBS) was from Oxoid (UK); Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), fatty acid-free bovine serum albumin (fBSA), gentamicin and L-glutamine were from Sigma–Aldrich (USA). Labeled (5,6,8,9,11,12,14,15-^{3}H)-AA (specific activity, 180–240 Ci mmol^{-1} (6.66×10^{12}–8.88×10^{12} Bq)) and the liquid scintillation cocktail OptiPhase Supermix were from NEN Perkin Elmer (USA). Deuterated methanol and dimethyl sulfoxide were used to prepare the NMR samples. 1D ^{1}H-NMR and ^{13}C-NMR, as well as standard 2D HMBC, COSY, NOESY, HSQC and HSQC-TOCSY experiments for structure elucidation of the isolated compounds were run on a Bruker AVANCE II instrument, operating at a proton frequency of 600.18 MHz and with a triple-resonance cryo probe, 5 mm, equipped with a z-gradient.

Plant material

Sclerochloa dura (whole plant) was collected in 2009 (June–July) near the Gabrovačka reka, Niš, Serbia. The identification of the plant was performed by Bojan Zlatković from the Department of Biology and Ecology, University of Niš, Serbia. A specimen of the identified plant was deposited in the Herbarium of the Faculty of Science and Mathematics (HMN) of the University of Niš, Serbia (voucher number 6922). The plant material was dried for 10 days in the dark, with proper ventilation and at room temperature. The dried plant was kept in a closed plastic bag in a dark at room temperature until extraction.

Extraction

Aerial plant parts (1.4 kg) were crushed into smaller pieces and extracted two times with methanol (5.0 L), each time for 24 h at room temperature. The extraction was aided by 90 min of sonication. The obtained extracts were filtered (Blue ribbon filter paper from Schleicher and Schuell) and combined. The volume was reduced to 500 mL on a rotary evaporator and the concentrated solution transferred into separation funnels. Extraction with *n*-hexane (three times, 400 mL) was used for defatting and the separated methanolic phase was dried on rotary evaporator. The weight of the dry methanolic extract was 170 g, giving a yield of 12 %.

Isolation and identification of bioactive fractions of S. dura

Isolation was realized using a multistage fractionation approach by liquid chromatography. Details of the isolation method are given in the Supplementary material to this paper.

Cell culture and treatment of SW982 cells

The human fibroblastlike synoviocyte cell line SW982 is used as a model system to determine PLA_2 activity by measuring AA release[19,26] SW982 cells are widely used as a model for rheumatoid synovial inflammation and express PLA_2 and cytokines after inflammatory stimulation by, *e.g.*, IL-1β. Cells were sub-cultured bi-weekly by routine trypsin detachment and kept in a sub-confluent state. The cells were cultured in DMEM supplemented with 10 % fetal bovine serum, 0.1 mg mL^{-1} gentamicin and 0.3 mg mL^{-1} L-glutamine, in a humidified carbon dioxide atmosphere at 37 °C. For the AA release experiments, 5×10^5cells per well were seeded in a 48-well per plate form. At two days post-confluency, the cells were labeled with ^{3}H-AA in serum-free DMEM overnight and processed at day three post-confluency to ensure differentiation and synchronization of the cells. The experiments were performed in serum-free DMEM in triplicate. Untreated cells without inducing agents or plant extract were included for non-stimulated controls; distilled water was added to these for vehicle control. The $cPLA_{2\alpha}$ inhibitor ATK was included as a positive control of inhibition.[15] Following treatments, the cells were routinely observed by microscopy to ensure unaltered cell morphology, integrity and viability.

AA release assay

The AA release assay determines the amount of AA released from cells under inflammatory conditions. In the presence of inhibiting compound(s), the AA release is reduced, which is taken as evidence that the compound(s) target some level in the arachidonyl cascade.

At two days post-confluency, the SW982 cells were serum-starved and labeled overnight with ^{3}H-AA at a concentration of 0.4 μCi mL^{-1} (148×10^8 Bq). Prior to the addition of *S. dura* fractions or ATK, the cells were washed with PBS containing fatty acid-free BSA at a concentration of 2.0 mg mL^{-1} to remove unincorporated radioactivity. Cells were pre-treated for 1 h or 2 h with various dilutions of fractions of the plant extract and ATK, respectively, prior to the addition of 10 ng mL^{-1} IL-1β to induce an inflammatory state (Fig. 2a). After 24 h of IL-1β stimulation, the supernatants were cleared of detached cells by centrifugation at 13000 rpm for 10 min. The adherent cells were dissolved in 1.0 M NaOH in order to measure the amount of labeled AA incorporated into the cells. The activities in the supernatants and cellular fractions were assessed by liquid scintillation counting in LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc., USA). The results are given as released ^{3}H-AA in the supernatants relative to total ^{3}H-AA incorporated into the cells. The inhibition was calculated as AA release % in inhibited relative to stimulated cells, normalized to the control and subtracted from 100.

RESULTS AND DISCUSSION

Several prefractions of the crude S. dura *extract inhibit AA release in a cellular bioactivity test model*

By means of MPLC, the crude extract was separated into 9 prefractions (A-I), which were tested for inhibitory effects in AA-release assay. Out of these, E, F and G were found to exert the highest inhibitory effect, as shown in Table I.

TABLE I. The inhibition activity of prefractions E, F and G. Experiments were performed in triplicate. The inhibition and IC_{50} values were based on two independent experiments. The IC_{50} could not be calculated for prefraction G, as a dose-dependent inhibition was not obtained

Prefraction	Concentration, mg mL^{-1}	Inhibition (mean±*SD*), %	IC_{50} / mg mL^{-1}
E	0.10	63.1±18.4	0.09
F	0.08	63.5±25.5	0.06
G	0.10	68.3±4.8	–

Prefraction E was temporarily given low priority for further investigation, because its screening by HPLC-DAD indicated the presence of mostly kaempferol and its derivatives (data not shown). Kaempferol compounds are already known by their anti-inflammatory activity[27] and therefore, attention was turned to prefractions F and G, as potential sources of new anti-inflammatory compounds.

By the bioactivity-guided study, from prefractions F and G, six compounds with pronounced anti-inflammatory activity were identified (Fig. 1).

Elucidation of the structure of the inhibitory compounds of S. dura

The isolated novel compound was identified as glycerol derivative (1-*O*-(3-*O*-linolenoyl-6-deoxy-6-sulfo-*α*-D-glucopyranosyl)glycerol). Further, five additional isolated compounds from the methanolic extract of *S. dura* with strong anti-inflammatory activity in the AA release assay included one more glycerol derivative (1-*O*-feruloylglycerol), three flavonoids (isovitexin, tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl) ether 7-*O*-*β*-glucopyranoside and tricin 7-glucoside) and one glucoside (byzantionoside B).

*Compound **1***. Compound **1** (Fig. 1, 38.8 mg) was isolated from the combined fractions G50 and G51 using semi-preparative HPLC (Table S-I of the Supplementary material). The analysis of spectral data revealed that compound **1** was a novel compound. HRMS indicated to a molecular formula $C_{27}H_{46}O_{11}S$ (ESI–) with a molecular mass of 578.2751 (theoretical value 578.2761). The analysis of the 1D and 2D NMR spectra with homo- and hetero-nuclear direct or long range correlations allowed structure elucidation and a complete ^{1}H and ^{13}C assignment, as given in the Supplementary material to this paper. The carbons 1–6 belonging to glucose moiety had shift values for ^{13}C ranging from δ 54.4 to 100.3 ppm and for protons from δ 2.92 to 4.77 ppm. The shift values of both ^{13}C and protons at C6 indicated a direct attachment to an SO_3H group, which was also confirmed by MS analysis. The 18 C-atoms with shift values ranging from δ 14.8 to 176.2 ppm with attached protons from δ 0.98 to 5.37 ppm belong to the linolenoyl chain of the molecule. An HMBC correlation peak of H3 (at δ 3.65 ppm) of glucose and carbon C1″ (δ 176.2 ppm) of the linolenoyl chain revealed the attachment position. The glycerol moiety exhibited shift values for carbons ranging from δ 64.4

to 72.7 ppm and protons from δ 3.36 to 4.05 ppm. The correlation peaks between carbon C1 of glucose and protons $H_{a,b}1'$ of glycerol in HMBC unambiguously revealed the connectivity between glucose and glycerol. The coupling constant of the H1 doublet of glucose referred to an axial position of the glycerol moiety. Both the molecular formula and NMR data indicated that **1** was 1-*O*-(3-*O*-linolenoyl-6-deoxy-6-sulfo-α-D-glucopyranosyl)glycerol (Fig. 1).

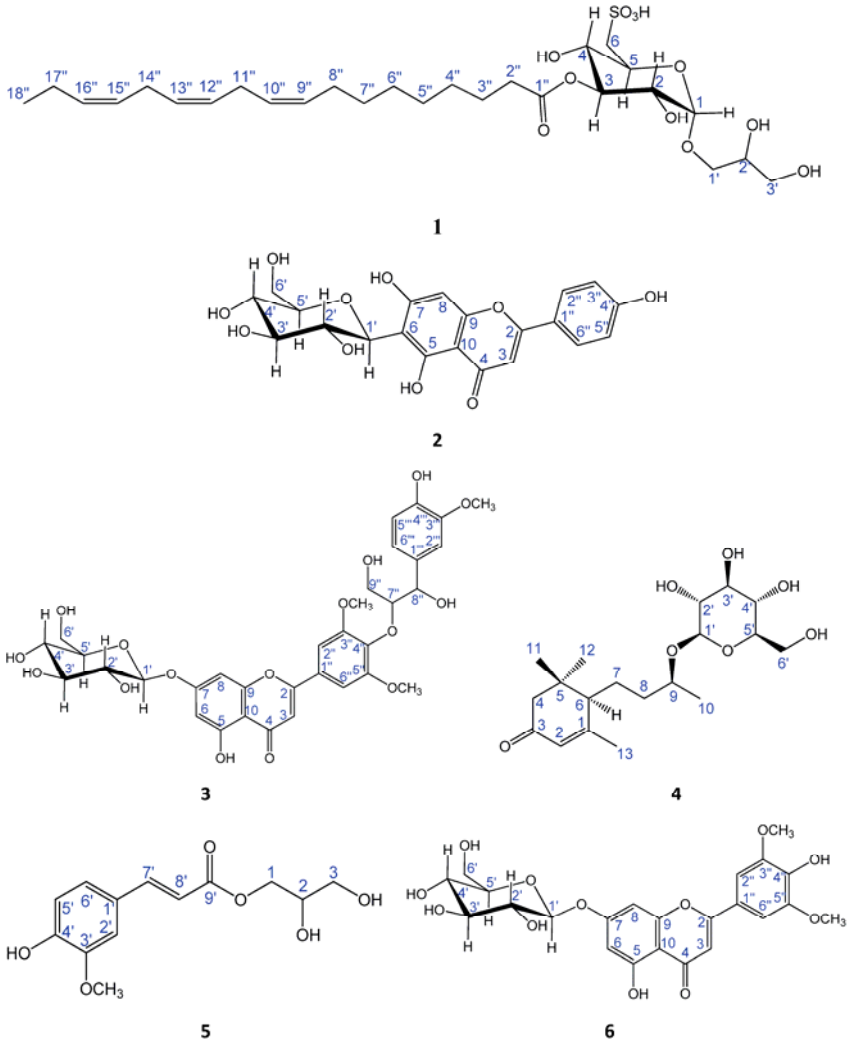


Fig. 1. The structures of the identified compounds from methanolic extract of *S. dura*. Compound **1**: 1-*O*-(3-*O*-linolenoyl-6-deoxy-6-sulfo-α-D-glucopyranosyl)glycerol; compound **2**: isovitexin; compound **3**: tricin 4′-*O*-(*erythro*-β-guaiacylglyceryl) ether 7-*O*-β-glucopyranoside; compound **4**: byzantionoside B; compound **5**: 1-*O*-feruloylglycerol; compound **6**: tricin 7-glucoside.

*Compound **2***. Compound **2** (14.3 mg) was identified as isovitexin (Fig. 1). It was isolated as the main compound in fraction F07 obtained in the preparative HPLC separation of prefraction F (Table S-II of the Supplementary material). HRMS (ESI+) was 432.1055, which corresponded to molecular formula $C_{21}H_{20}O_{10}$ (theoretical value 432.1056). The signal assignments for carbons and protons can be seen in Supplementary material. The NMR data were in agreement with those already published.[28]

*Compound **3***. Compound **3** (17.0 mg), Fig. 1, the main compound in fraction F51, was obtained by preparative HPLC separation of the prefraction F (Table S-II). It was identified as tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl) ether 7-*O*-*β*--glucopyranoside. The found value of HRMS (ESI+) was 688.2003 (theoretical value 688.2003), which corresponded to the molecular formula $C_{33}H_{36}O_{16}$. The NMR data of the compound (Supplementary material) fit the published ones.[29]

*Compound **4***. Semi-preparative HPLC separation of groups F40–F44 (Table S-III of the Supplementary material to this paper) yielded 34.3 mg of compound **4** (Fig. 1). The HRMS (ESI+) value for **4** was 372.2147 compared to the theoretical value 372.2148, which matches the molecular formula $C_{19}H_{32}O_7$. Compound **4** was identified as byzantionoside B. The NMR data (Supplementary material) were in agreement with those published previously.[30]

*Compound **5***. The semi-preparative HPLC separation of groups F70–F74 (Table S-III) gave 39.6 mg of compound **5** (Fig. 1). It was identified as 1-*O*-feruloylglycerol. The HRMS (ESI+) value was found to be 268.0952 (corresponding to the molecular formula $C_{13}H_{16}O_6$), with the theoretical value 268.0947. The NMR resonances of ^1H and ^{13}C for **5** (Supplementary material) were in agreement with those reported previously.[31]

*Compound **6***. A total of 15.5 mg of compound **6** (Fig. 1) was obtained as the main compound of fraction F-32, by preparative HPLC separation of prefraction F (Table S-II of the Supplementary material). It was identified as tricin 7-glucoside (Fig. 1). The theoretical MS (ESI+) value for $C_{23}H_{24}O_{12}$ is 492.1268 and 492.1263 was found. The NMR data of **6** (Table S-IV of the Supplementary material) corresponded to those reported previously.[32]

Comparison of bioactivity of the identified compounds from S. dura

The respective fractions containing each of these compounds were again tested for their bioactivity to confirm inhibitory effects on the release of arachidonic acid, thus implying that the PLA_2 enzyme activity is affected. The identified compounds from *S. dura* inhibited the IL-1*β*-induced release of AA in SW982 cells in the following order of decreasing efficiency: **5**>**4**>**1**>**3**>**6**>**2**.

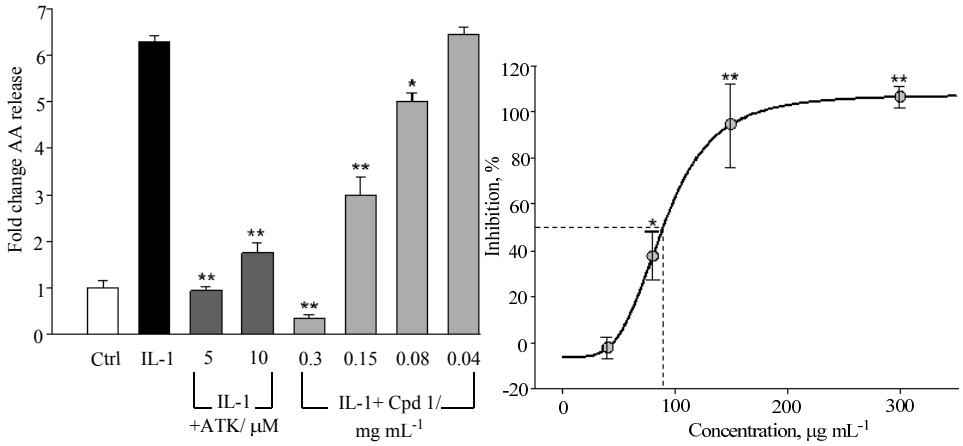
Based on the observed unchanged morphology (data not shown), all the compounds were well tolerated by the SW982 cells, including the novel compound **1**. To characterize more closely the inhibitor potency, a series of repeated

dose-response experiments were performed, which led to an estimated IC_{50} of 0.09 mg mL^{-1} (Table II and Fig. 2b).

TABLE II. Comparison of the activity of compounds **1**–**6** based on experiments performed in triplicate

Compound	Concentration, mg mL^{-1}	Inhibition, % (mean ± S.D.)
1	0.15	92.8±46.52
2	0.30	26.8±35.60
3	0.15	67.3±7.10
4	0.12	96.9[a]
5	0.09	99.5[a]
6	0.30	54.9±41.40

[a]Based on one experiment due to limited amounts of compound



(a) (b)

Fig. 2. a) Compound **1** inhibits cellular AA release in a dose-dependent manner following 24 h stimulation with IL-1β (10 ng mL^{-1}). The cPLA$_{2\alpha}$ specific inhibitor ATK is included for reference. b) Estimated IC_{50} for compound **1** is 0.09±0.03 mg mL^{-1} (dotted lines). The results shown are means ± *SD* of experiments 2–5 performed in triplicate. * p<0.05, ** p<0.001 *vs*. IL-1β alone (no inhibition).

As the molecular weight of compound **1** is 578.2751 g mol^{-1}, the mole percentage of **1** in the active fraction was estimated to be 60 %, based on the integral ratio in the ^{1}H-NMR spectrum. Hence, the estimated IC_{50} of 0.09±0.03 mg mL^{-1} corresponds to 114.1±31.1 μM. As expected, the cPLA$_{2\alpha}$ specific inhibitor ATK is far more potent, with reported IC_{50} values of 2 μM and 8 μM in inhibiting AA release from platelets and monocytes, respectively. Taken together, these results indicate that compound **1** may have potential as a novel anti-inflammatory compound, interfering with and normalizing the available levels of pro-inflammatory arachidonic acid.

CONCLUSIONS

In this study, one novel compound together with five known compounds was isolated and identified from the methanolic extract of the aerial parts of *Sclerochloa dura*. All isolated compounds had the ability to inhibit the PLA_2 activity as assessed by their ability to inhibit cellular AA release and, therefore, could be considered as lead compounds for the development of potent anti-inflammatory drugs. To the best of our knowledge, this is the first report that these compounds may inhibit of PLA_2 activity at some level, a finding that requires further investigation. It is still unclear if the compounds affect kinases or other effectors upstream of PLA_2, if they inhibit PLA_2 enzymes, or if the compounds affect the receptors at the cell surface. To help clarify such issues, work is now in progress to establish the synthesis of the novel compound and its derivatives.

SUPPLEMENTARY MATERIAL

Details concerning the isolation and identification of the bioactive fractions of *S. dura* and the assignments of the ^{1}H- and ^{13}C-NMR resonances of compounds **1**–**6** are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

Acknowledgements. Funding of this research was provided by HEC Pakistan *via* SIU Norway, Discovery-NTNU/TTO and MedTech Trondheim AS Foundation. The experimental work was realized at the Institute of Chemistry and Department of Biology, NT Faculty, Norwegian University of Science and Technology, Trondheim, Norway. Technical assistance was provided by Dr Asma Zaidi.

ИЗВОД

НОВА ЈЕДИЊЕЊА ИЗОЛОВАНА ИЗ *Sclerochloa dura* ИМАЈУ АНТИИНФЛАМАТОРНО ДЕЈСТВО

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Активирање PLA_2 посредством проинфламаторних цитокина узрокује ослобађање арахидонске киселине (AA) и стварање еикозаноида који изазивају упалу. Нестероидни лекови против упала, који блокирају циклооксигеназу (COX1/2) одговорну за ензимску трансформацију AA у еикозаноиде, успешно делују против болова и тескоба узрокованих упалама. Коришћењем теста којим се ослобађа AA као путоказа за утврђивањем биолошке и антиинфламаторне активности, изоловано је, из метанолног екстракта надземних делова *Sclerochloa dura*, ново једињење 1-*O*-(3-*O*-линоленоил-6-деокси-6-сулфо-*α*-D-глукопиранозил)-глицерол (**1**), заједно са још пет познатих једињења: изовитексин, бизантинозид Б, трицин-4′-*O*-(*еритро*-*β*-вајацилглицерил)-етар-7-*O*-*β*--глукопиранозид, 1-*O*-ферулоил-глицерол- и трицин-7-глукозид, која су окарактерисана

на исти начин. Једињења су изолована хроматографским техникама (Sephadex LH-20 хроматографија на колони, HPLC, препаративни и семипрепаративни HPLC) и окарактерисан спектроскопским техникама (НМР спектроскопија и масена спектрометрија). Инхибиторна активност ослобађања АА новог једињења **1** има IC_{50} = 0,09±0,03 mg mL^{-1}.

(Примљено 19. јануара, ревидирано 26. јуна, прихваћено 28. јуна 2015)

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