The Natural Products Journal, **2025,** *15,* **e22103155334830**

RESEARCH ARTICLE

Phytochemical Screening of Genus Primula Species Growing in Georgia and Study of their Antioxidant and Anti-inflammatory Potentials

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Abstract: ***Background*:** In traditional medicine, species of the genus *Primula* L. are used to treat various health conditions, such as eye disorders, respiratory infections, headaches, epilepsy, insomnia, as expectorants, and to promote wound healing.

***Objective*:** The goal of this article was to evaluate the antioxidant and anti-inflammatory activities of three species from genus *Primula* L. growing in Georgia: *Primula macrocalyx*, *Primula woronowii* and *Primula saguramica*.

***Methods*:** Initially, fractions containing both aerial and underground parts were air-dried, ground, and extracted with 80% ethanol. The extract was then concentrated by condensation and further dried through freeze-drying. Subsequently, additional chromatographic separations were carried out on Diaion HP-20 using solvents such as water, methanol (50% and 100%), and 100% ethyl acetate to isolate the desired fractions. To identify flavonoids and triterpene glycosides, the study employed thin-layer chromatography (TLC) alongside preliminary phytochemical tests. The antioxidant activity of these species was estimated *in vitro* by cell-free systems using ABTS and DPPH assays. The extracts' anti-inflammatory properties were evaluated using an *ex-vivo* cell system that isolated neutrophils. The study examined the extracts' impact on reactive oxygen species (ROS) production in neutrophils stimulated with PMA, as well as their effect on the catalytic activity of myeloperoxidase (MPO), a marker of inflammation in neutrophils.

***Results*:** Flavonoids and triterpene glycosides were primarily identified in the 50% and 100% methanol (MeOH) fractions of Primula species through TLC and preliminary phytochemical tests. For each experiment, gallic acid and quercetin served as standards at a concentration of 1 mg/ml, while the tested samples were prepared at concentrations of 5 mg/ml. Based on the IC_{50} findings, P. w 3 exhibits the most potent antioxidant and anti-inflammatory properties, as evidenced by the following indicators: ABTS - IC_{50}=8.51 ± 0.18; DPPH - IC_{50}=34.57 ± 0.47; PMN - IC_{50}=0.68 ± 0.04; SIEFED - IC_{50}=1.49 ± 0.7; and classical IC_{50}=1.89 ± 0.01.

***Conclusion*:** Among the tested fractions, only the fraction of the 3 species prepared in MeOH (50%) showed the best dose-dependent antioxidant and anti-inflammatory activities, especially P.w 3 which is probably related to the high flavonoid content found in this species.

Keywords: *Primula macrocalyx, Primula woronowii, Primula saguramica,* stoichiometric anticatalytic, antioxidant, ABTS, DPPH, neutrophils, myeloperoxidase, reactive oxygen species stoichiometric.

ARTICLE HISTORY

Received: July 05, 2024
Revised: August 01, 2024
Accepted: August 09, 2024

DOI:
10.2174/0122103155334830240912072007

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2210-3163/25 $65.00+.00

1. INTRODUCTION

Plants have been utilized in traditional medicine systems for millennia, given their extensive repertoire of bioactive compounds with therapeutic potential. The vast diversity of plant species presents a considerable opportunity for the discovery of novel pharmacological agents. Additionally, the production of plant-derived medicines can be more cost-effective compared to the synthesis of pharmaceuticals, thereby enhancing the accessibility of treatments, particularly in settings with limited resources [1]. The flora of Georgia is among the richest in countries with temperate climates. Consequently, it is important to study not only the plant species that grow in Georgia but also its endemic species [2]. Our research focuses on species belonging to the family Primulaceae, which includes 59 genera of plants and approximately 697 species [3, 4]. Primulas are native to various regions, including the tropical mountain areas of Ethiopia, the temperate zones of the northern hemisphere, Indonesia, New Guinea, and South America [5]. In Georgia, this family is represented by 7 genera and 46 species. Twenty-two species of the genus *Primula* L. can be found in Georgia, among them, 10 are endemics of the Caucasus, and two - endemics of Georgia: *Primula abchasica* and *Primula saguramica* [4]. Phytochemical investigation of various species of the genus Primula L. revealed that flavonoids are widely distributed in this genus [6-8]. Additionally, the primary bioactive constituents of Primula flowers and roots include triterpene saponins, phenolic acids, and phenolic glycosides [9, 10]. Numerous studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals [11]. It is recognized that reactive oxygen species (ROS) play different roles *in vivo*. Some of these effects are beneficial and connected to cell growth as well as to intercellular signaling. However, high ROS production may be very harmful since they can oxidize lipids, cell membranes, proteins in tissues, enzymes, carbohydrates, and DNA [11]. The antioxidant abilities of flavonoids can help to decrease these harmful effects. The hydroxyl groups of aromatic rings in flavonoids donate the H atom to various radicals, such as peroxyl, hydroxyl and other radicals, which lose reactivity due to their stabilization and, on the other hand, form a relatively stable flavonoid radical [12]. Furthermore, the presence of C-2 and C-3 double bonds, along with hydroxyl (OH) groups at the C-3 position of flavonoids, enhances the molecule's ability to stabilize electrons through resonance, thereby contributing to superior antioxidant properties [13]. Several enzymes involved in inflammation, including xanthine oxidase (XO), cyclooxygenase (COX), lipoxygenase phosphoinositide 3-kinase, and myeloperoxidase (MPO), are also known to be potently inhibited by flavonoids [14, 15]. On the other hand, Saponins are a varied group of naturally occurring plant secondary metabolites. They feature a hydrophilic sugar component attached to a lipophilic aglycone, which gives them amphiphilic characteristics and distinctive functional properties [16]. Saponins are utilized across the pharmaceutical and related fields for their advantageous effects [17]. They may help lower blood lipid levels, decrease cancer risk, and regulate blood glucose levels. A diet rich in saponins is thought to aid in preventing dental decay and reducing platelet aggregation. Furthermore, saponins show promise in treating hypercalciuria and counteracting acute lead poisoning [18]. Saponins are also used as agents to promote expectoration and relieve coughs [19]. In the cosmetics industry, saponins are prized for their natural ability to emulsify, foam, and cleanse. They are used in a range of products, including shampoos, soaps, facial cleansers, body washes, and shaving creams, where they enhance lathering, cleaning, and moisturizing properties [20]. In addition, bis-bibenzyls compounds were isolated from some species of Primula: Riccardin-C was extracted from the rhizomes of P. veris, which showed pronounced anti-cancer potential [21]. In traditional medicine, different plant species of the genus Primula have been used from immemorial times by the inhabitants of Greece as an antidote to snake poison, and their juice was applied to relieve toothache [22]. Some species of the genus are used traditionally to treat epilepsy and convulsions [23]. Different species of the genus promote the functioning of the liver and spleen and remove obstructions of these organs; their constituents have been prescribed to relieve renal pain, used to cure boils, and scorpion strings, and are also used as sedatives [24]. In Denmark, Primula flowers are used to make distilled water, and infusions from either fresh or dried plants are utilized to treat headaches, epileptic seizures, and insomnia. Additionally, these plants are incorporated into traditional medicine practices to support the healing process [25]. In traditional folk medicine, extracts from the leaves and roots of Primula vulgaris are employed to address skin issues, ulcers, and support wound healing [26]. *Primula macrocalyx* has been extensively utilized in traditional medicine for its expectorant, diuretic, sedative, spasmolytic, and sudorific properties. It is used to address a range of conditions, including vitamin deficiencies, colds, fevers, headaches, insomnia, paralysis, scurvy, tuberculosis, heart disease, rheumatism, and kidney disorders [27, 28]. In addition, Primula finds applications in both the culinary and cosmetic fields. The flowers are utilized as decorative, edible additions to salads, desserts, and beverages, while the oil derived from its seeds is highly valued in the cosmetic industry for its benefits to skin and hair. Additionally, scientific studies have investigated Primula's genetic variety, its role in ecosystems, and its possible impact on adapting to climate change [29]. In Georgian folk medicine, Primula species were used for cough, especially in children's practice with boiled grape juice [30]. Zaza Panaskerteli's book, "Karabadine'' mentions the Primula species flower with a mixture of honey and vinegar as a wound healer [31]. Primula species are renowned for their diverse biological activities. Extracts from these plants are commonly utilized in the treatment of respiratory tract infections [32]. The saponins present in Primula exhibit potent antifungal activity against Candida species [33]. Notably, the flower extract of *Primula vulgaris* has demonstrated significant anticancer effects against HeLa cells by disrupting the cell cycle at the S phase and increasing the rate of apoptosis. This effect, which is concentration-dependent, also involves a decrease in mitochondrial membrane potential, akin to the response observed in standard fibroblast cells [34]. In traditional medicine, leaf infusions from various Primula species are employed as diuretics for kidney and bladder disorders and as analgesics for rheumatic conditions.

Conversely, flower infusions are used for a variety of therapeutic purposes, including as diaphoretic expectorants and anti-inflammatories for bronchitis, insomnia, tachycardia, migraines, and nervousness [25]. Aqueous and ethanolic extracts of *Primula elatior* and *Primula veris* exhibit potent, dose-dependent effects in managing epilepsy and convulsions, and they also enhance tranquility. This efficacy is evident in flumazenil-binding assays that target benzodiazepine receptors [35]. The compound Primin, isolated from *Primula obconica*, demonstrates both antibacterial and anticancer properties [36]. Additionally, *Primula denticulata* is notable for its antioxidant, antidiabetic, and antileishmanial activities. Traditionally, the flower extract of this species is used to address eye-related issues [37, 38]. Furthermore, Sitosterol glucoside from *Primula macrophylla* also shows antileishmanial effects [39]. Omega-3 polyunsaturated fatty acids, integral components of Primula species, contribute positively to cardiovascular health [40]. Lastly, saponins derived from *Phaseolus vulgaris* and *Cyclamen persicum*, when diluted to a 1:1000 ratio, exhibit spermicidal properties, demonstrating a natural ability to immobilize and kill sperm [41]. Given this diverse pharmacological activity, we are particularly interested in *Primula macroxalyx*, *Primula woronowii* being endemic in the Caucasus, and *Primula saguramica* being endemic in Georgia. Although the population of Georgia actively uses these plants as food and for the treatment of various diseases, such as avitaminosis, cough, *etc*., it is important to understand their biological action mode in relationship with their content of secondary metabolites [42]. Different fractions of these plants were obtained from the crudes, and using an increasing gradient (0-100%) of MeOH in water or ethyl acetate (EtOAc) was used to separate compounds with different polarities. In the literature, few data concern the anti-inflammatory potential of these species. Polymorphonuclear neutrophils (PMNs) play a key role in inflammatory response. PMNs are the major component of phagocytic cells in the human bloodstream. They were stimulated and undergo a respiratory burst when they phagocyte foreign agents as bacteria and damage cells [43]. The particles that are engulfed are subjected to a substantial amount of superoxide anion molecules generated by the NADPH-oxidase complex. Once the phagocytic vacuole is formed, fusion with other granules in the neutrophil cytoplasm releases myeloperoxidase (MPO), which uses hydrogen peroxide as a substrate and oxidizes chloride to hypochlorous acid, a strong oxidant molecule that can oxidize in turn many biological molecules, and reduce thiol groups. This leads to serial events resulting in bacterial killing [43]. In cases of severe inflammation, there is significant, uncontrolled activation of polymorphonuclear leukocytes (PMNs), resulting in increased production of reactive oxygen species (ROS) and the release of large quantities of myeloperoxidase (MPO), which is considered a pro-oxidant biomarker in this context. This process triggers and amplifies oxidation, nitration, and chlorination of molecules in the extracellular environment. These detrimental effects can also impact important molecules such as DNA, neurotransmitters, and proteins, potentially leading to serious diseases. One strategy to mitigate these harmful effects involves reducing the excessive production of ROS and lowering MPO activity [43]. In our study, we examined the antioxidant capabilities of fractions using four distinct methods: (i) a cell-free system employing ABTS and DPPH assays to gauge how extract molecules interact with and counteract free radical production; (ii) a chemiluminescent assay employing a cellular model to evaluate how extracts influence the regulation of reactive oxygen species (ROS) production by stimulated neutrophils; (iii) an innovative pharmacological approach (SIEFED) to investigate the extracts' ability to inhibit MPO activity by targeting its active site; and (iv) a pharmacological method to assess the extracts' potential as electron donors in the peroxidase cycle of MPO.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

2,2-Azinobis-(3-ethylbenzohiazoline-6-sulphonic acid (ABTS) was from Fluka (Bornem, Belgium). Sodium and potassium chloride, ethanol, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), Tween 20 were analytical-grade products from Merck (VWRI, Leuven, Belgium). L-012 (8-amino-5-chloro-7-phenyl-pyrido[3,4-d] pyridazine-1,4(2H,3H)dione) was obtained from FujiFilm Wako Chemical Europe (Neuss, Germany) were Amplex Red (10-acety l-3,7-dihydroxyphenoxazine) was purchased from Invitrogen Phorbol 12-myristate 13-acetate (PMA), gallic acid (2,4,6-trihydroxybenzoic acid) and sodium persulfate ($Na_2S_2O_8$) DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Aldrich (Steinheim, Germany). Quercetin (3,3,4,5,7-pentahydroxy-2-phenylchromen-4-one) was from ChromaDex (LGC Standard, France). De-oxygenated milliQ water or ultrapure water (EasyPure UV purification system) was used for the preparation of all solutions. Fluoroskan Ascent FL (Fisher Scientific, Tournai, Belgium) and Multiskan Ascent (Thermo Labsystem) were used for the determination of antioxidant activity.

2.2. Sample Collection

The following plants are the research objects from the genus Primula. L.: *Primula macrocalyx* Bunge., was collected in the floristic area of Kartli, in the meadows of the Saguramo-Zedazeni forest-park, in the forest edges E044.788210, at H-990m, in the active flowering phase in 2021, *Primula woronowii* Losinsk. was collected in the floristic area of Kartli, in the meadows of the Saguramo-Zedazeni forest-park, E044.785340, at H-891m, in the active flowering phase in 2020 and *Primula saguramica* Gavr. was collected in the floristic area of Kartli, on the eastern slope of the Tbilisi ridge, on the slopes of village Karsan in oak-shrub forests E044.712020, at H-798m, in active flowering phase in 2021 [4].

2.3. Extraction Procedure and Preliminary Phytochemicals Screening

The first step was the selection of extraction conditions: A shoot and root of all species were dried, grinded and three-time extraction with 80% EtOH was performed. After condensation with a rotary evaporator at 70°C, crude extracts were obtained with lyophilization (P.m 1, P.w 1, P.s 1). From the crude extracts, the following enriched fractions were obtained by column chromatography on Diaion HP-20, with an increasing gradient: H_2O – P.m 2, P.w 2, P.s 2,

MeOH 50% - P.m 3, P.w 3, P.s 3, MeOH 100% - P.m 4, P.w 4, P.s 4, EtOAc 100% - P.m 5, P.w 5, P.s 5 (Fig. **1**).

The ethanol 80%, water, methanol 50%, methanol and ethyl acetate extracts (1 g) were dissolved in 100 mL of their own mother solvents for preparing the stock solution, and the latter was used for phytochemical screening following the methodology of Harborne and Kokate [44, 45]. Thin layer chromatography (TLC) was performed to screen for secondary metabolites [20]. In both cases, flavonoid and triterpene glycosides were revealed. For TLC of flavonoids and triterpene glycosides, the system, $CHCl_3$: MeOH: H_2O - 26:14:3 was used [46].

2.4. ABTS (2,2-azinobis-(3-ethylbenzohiazoline-6-sulphonic Acid) Free Radical Scavenging Activity Assay

The first ABTS assay was described in 1993 by Miller [47]. The method was developed based on the absorbance of the ABTS.+ radical cation for the evaluation of the total antioxidant capacity of body fluids and drug solutions. This method was first based on the production of ABTS radical cation by activation of metmyoglobin with hydrogen peroxide to generate ferryl myoglobin radical. The latter reacted with ABTS to form the $ABTS^{\circ+}$. ABTS radical cation can alternatively be produced by the reaction between ABTS and sodium persulfate, resulting directly in the formation of the blue/ green ABTS°+ chromophore [48, 49]. The ABTS free radical-scavenging activity of each extract was measured with a Multiskan Ascent plate reader at 740 nm. The starting concentrations of gallic acid and quercetin were 1 mg/ml and the final ranged from 10 to 0.625 µg/ml. The starting concentrations of tested samples were 5 mg/ml and the final ranged from 50 to 3.125 µg/ml. The samples and standards were dissolved in DMSO and distributed in 96-well UV-Transparent Microplates control consisting of 0.02 mL DMSO in 1.98 mL of ABTS solution. The measurements were performed three times. A control consisted of 0.02 mL DMSO in 1.98 mL of ABTS solution. Samples were prepared and measured in triplicates.

2.5. DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical Scavenging Activity Assay

The DPPH assay was initially proposed in the 1950s to identify electron donors within natural products [50]. Subsequently, it has been employed to assess the antioxidant properties of phenolic compounds and plant-based foods [51]. DPPH (2,2-diphenyl-1-picrylhydrazyl) is an organic nitrogen radical characterized by an unpaired electron located at one of its nitrogen atoms. It exists in a stable, soluble organic form and appears purple in color in its insoluble aqueous medium. The DPPH free radical-scavenging activity of each extract was measured with a Multiskan Ascent plate reader at 510 nm [52]. The starting concentrations of gallic acid and quercetin were 1 mg/ml and final ranging from 10 to 0.625 µg/ml. The starting concentrations of tested samples were 5 mg/ml and the final concentrations ranged from 50 to 3.125 µg/ml. The samples and standards were dissolved in DMSO and distributed in 96-well UV-Transparent Microplates. The control sample comprised 0.02 mL of DMSO diluted in 1.98 mL of DPPH solution. The measurements were performed three times.

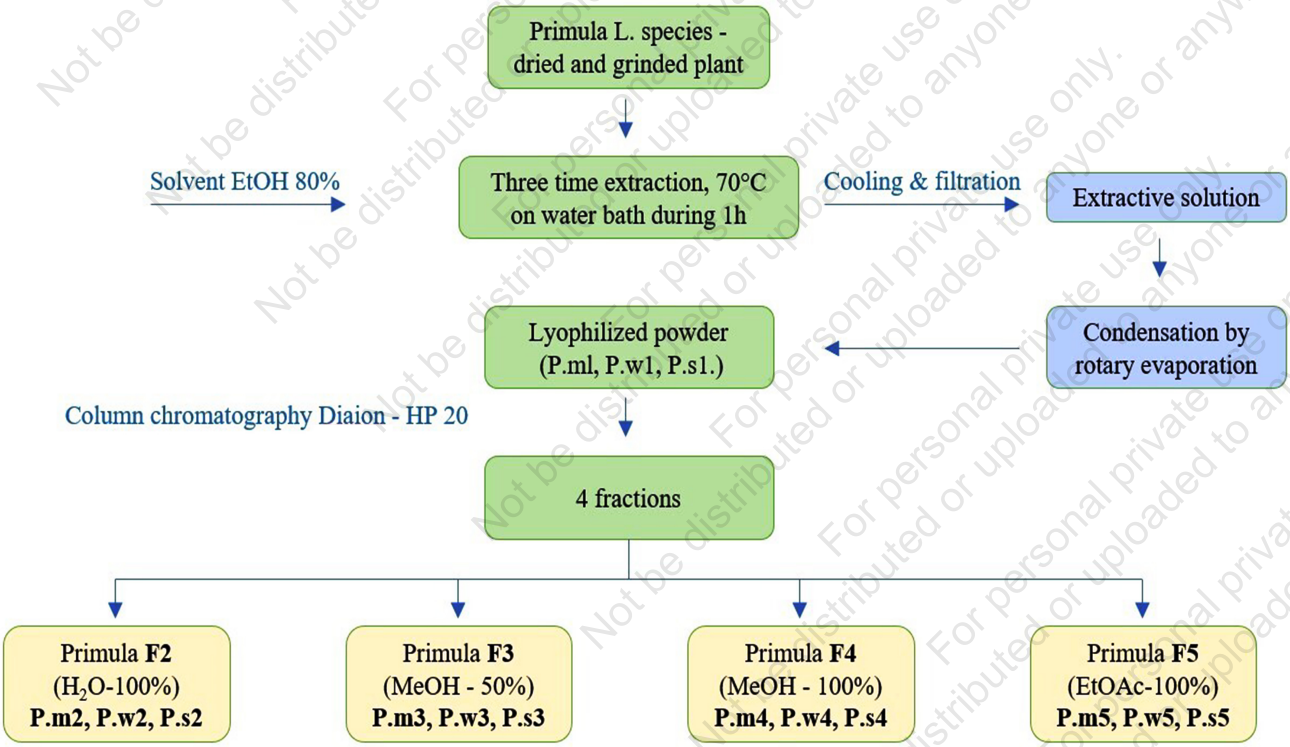

Fig. (1). Extraction procedure and sampling of *Primula species* fractions. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2.6. Measurement of Reactive Oxygen Species (ROS) Production

ROS production by activated neutrophils was assessed using L-012-enhanced chemiluminescence (CL), following the methodology adapted from Benbarek *et al*. [53] and Franck *et al*. [54]. Phorbol 12-myristate 13-acetate (PMA) was used for neutrophil stimulation. The latter is commonly used to study the neutrophil because PMA induces pathways to other stimuli, with the participation of PKC, MPO, and neutrophil elastase (NE). Also PMA induces neutrophil degranulation, metabolic changes, ROS, and NET formation [55, 56]. 10 µl of L-012 and 10 µl of PMA. The plate was incubated for 10 min at 37°C with fractions. The CL response of the neutrophils was monitored for 30 min at 37°C with a Fluoroskan Ascent FL (Fisher Scientific, Tournai, Belgium) and expressed as the integral value of the total CL emission. A control assay set as 100% of CL response was performed with PMA-stimulated neutrophils where an equivalent volume of DMSO solution was added instead of the samples. Samples were prepared and measured in triplicates [57]. The starting concentrations of gallic acid and quercetin were 1 mg/ml and final ranging from 10 to 0.019 µg/ml. The starting concentrations of tested samples were 5 mg/ml, and final concentrations ranged from 50 to 0.78 µg/ml

2.7. The SIEFED ("Specific Immunological Extraction Followed by Enzymatic Detection") and Classical Enzymatic Methods for Measurement of Equine Active Myeloperoxidase in Biological Samples

Myeloperoxidase (MPO) is an abundant heme-containing enzyme which is mostly present in neutrophils. In response to an invading agent in the body, MPO produces hypochlorous acid (HOCl), which damages various cellular structures. Therefore, it is important to determine the anticatalytic activity of secondary metabolites. For the determination MPO inhibitory activity, SIEFED (specific immunological extraction followed by enzymatic detection) method was performed. This method is specific because cobaye anti-MPO IgG antibodies (3 µg/ml) against MPO were bound into the wells of a microplate to capture MPO. 150 microliters of MPO solution were placed in a 96-wells microtiter plate (Combiplate 8 EB) from Thermo Scientific (Breda, Netherlands), coated with cobaye anti-human MPO polyclonal antibodies (CORD, Belgium) following 3 microliters of samples and 147 microliters of PBS. All plates were kept in darkness and incubated at 37°C for 2 hours before the wells were washed. Following the washing step, the peroxidase activity of MPO was assessed using a fluorogenic substrate, hydrogen peroxide, along with nitrite as a reaction enhancer [58]. For the classical enzymatic assay, this method reveals the electron-donating potential of a molecule in MPO peroxidase cycle. Unlike the previous method, specific antibodies are not used here, washing and incubation steps are not performed. Fluorescence was measured with a Fluoroscan Ascent plate reader at the excitation and emission wavelengths of 544 and 590 nm, respectively. The fluorescence value was directly proportional to the quantity of active MPO present in the sample [58]. A control assay, established with purified MPO solution, was set to represent 100% MPO activity. In this control, an equivalent volume of DMSO solution was added instead of the test samples.

2.8. Statistical Analysis

For ABTS, DPPH, SIEFED and CL assays, within an experiment, each assay was conducted at least three times. The statistical analysis was performed with GraphPad Instat 3.05 (GraphPad Software, San Diego, CA, USA). All results are presented as mean ± standard deviation (SD) and expressed as percentages relative to the controls, which were normalized to 100% (supplementary material).

3. RESULTS

3.1. Extraction Procedure and Preliminary Phytochemicals Screening

In the first step, for screening of some secondary metabolites in Primula species, it was important to perform thin layer chromatography (TLC) and preliminary phytochemical tests [44-46]. According to the results, the content of flavonoids and triterpene glycosides was determined (Supplementary material Figs. **1A-C**). As a reference, standard rutin was used at a starting concentration of 1 mg/ml and samples with initial concentrations of 20 mg/ml. In advance, for TLC, there was prepared system - $CHCl_3$: MeOH: H_2O ratio-26:14:3 and silica gel plate was placed in the chromatography tank after about 2 hours. For the revelation of secondary metabolites (flavonoids and triterpene glycosides), 1% 2-aminoethyl diphenylborinate and 1% vanillin solution were used, respectively. According to the results, flavonoids were characterized for 1, 3 and 4 fractions for all species; more intense color seems to be detected for P.m3, P.w3 and P.s3. On the other hand, triterpenes mostly appear in 1 and 4 fractions.

3.2. Impact of the Fractions on Scavenging Activity against ABTS*+ Radical Cation and DPPH* Formation

ABTS and DPPH anti-radical assays were used for the stoichiometric activity assessment of all the fractions. Gallic acid and quercetin were used as control standards and were more efficient than fractions. The majority of the tested fractions demonstrated a dose-dependent radical scavenging activity except fractions obtained in H_2O and EtOAc. From these results, a calculation of IC_{50} was performed (Table **1**). The best scavenging activity was found for the standards gallic acid and querectin followed by the plant fractions Pm, Pw, and P.s prepared in MeOH (50%). The fraction from P.w showed the best results (P.w 3 ABTS - IC_{50}=8.51 ± 0.18; DPPH - IC_{50}=34.57 ± 0.47). Results from ABTS indicate that the reaction between the studied fraction and ABTS radical species is faster and better than for DPPH assay (Figs. **2A-C**). For some fractions (P.m1, P.m2, P.m4, P.m5; P.w 1DPPH, P.w2, P.w4 DPPH, P.w5; P.s1, P.s2, P.s4 P.s5), the IC_{50} could not be determined, because at these concentrations, the inhibition of free radicals (ABTS, DPPH) in percentage, did not exceed 50%.

3.3. Effect of the Fractions on ROS Production by Stimulated Neutrophils

One of the most important mechanisms for the determination of anti-inflammatory activity is to study the inhibition

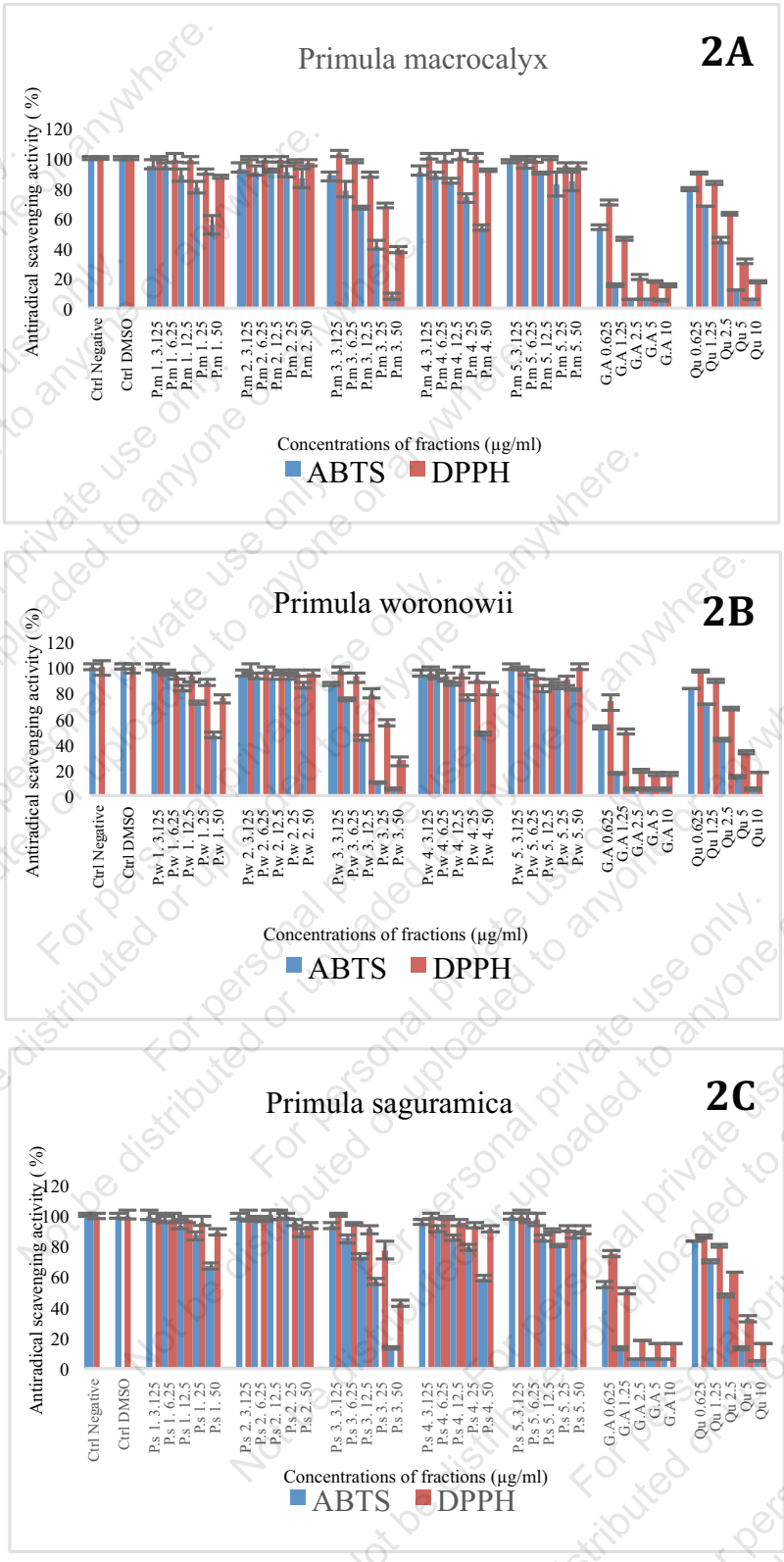

Fig. (2). Relative ABTS and DPPH radical scavenging capacity derived from *Primula macroxalyx* (P.m.) (**A**), *Primula woronowii* (P.w) (**B**) *Primula saguramica* (**C**) fractions obtained after ethanol extraction and freeze-drying (1) or after chromatographic separation with H_2O, 50 % MeOH, 100 % MeOH and EtOAc (fractions 2, 3, 4, and 5, respectively). Gallic acid (GA) and quercetin (Qu) were used as reference compounds. All the extract powders were solubilized in DMSO and their scavenging capacity was compared to DMSO control (Ctrl DMSO) and ABTS or DPPH solution without sample (Ctrl negative). Results were obtained after 30 min incubation time and expressed in relative percentages vs Ctrl negative set as 100 % (Mean +/- SD, n=3 independent experiments with 3 technical replicates for each experiment). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

of ROS produced by stimulated neutrophils. Gallic acid used as control standard was more efficient than fractions. Considering the 3 species, fractions 3 and 4 prepared in MeOH (50%) and MeOH (100%), respectively showed the best results. The fractions obtained with ethanol extraction and freeze-drying (fraction 1) also showed good results but were less effective than for 3 and 4 fractions Fig. (**3**). For plant fractions 3 and 4, additional dilutions were prepared for the determination of IC_{50} (Supplementary material Figs. **2A-C**). Based on these experiments, IC_{50} was calculated and the best one was determined for: P.w 3 PMN - IC_{50} 0.68 ± 0.04 (Table **1**). For some fractions, the IC_{50} could not be determined because at these concentrations, the inhibition of ROS in percentage did not exceed 50%.

Supplementary material Fig. (**2**) Relative inhibition of neutrophils ROS production induced by *Primula macroxalyx* (P.m.) (**A**), *Primula woronowii* (P.w) (**B**) nd *Primula saguramica* (**C**) fractions obtained after chromatographic separation with 50 % MeOH and 100 % MeOH (fractions 3 and 4). Gallic acid (GA) and quercetin (Qu) were used as reference compounds. All the powdered extracts were solubilized in DMSO and their ROS inhibition capacity was compared to DMSO control (Ctrl DMSO) and stimulated neutrophils without sample (ST PMN). Results were obtained after 30 min incubation time and expressed in relative percentages vs. negative Ctrl set as 100 % (Mean +/- SD, n=3 independent experiments with 3 technical replicates for each experiment).

3.4. Effect of the Fractions on MPO Activity

Flavonoids are compounds with different numbers of hydroxyl groups. In addition to their antioxidant activity, another significant pharmacological effect related to inflammation is their capability to influence MPO activity. This can occur either by entering and obstructing the enzyme's active site or by serving as electron donors in the peroxidase cycle of the enzyme. These abilities were determined by the SIEFED and the classical assay of the enzyme, respectively. First reference molecules, gallic acid and quercetin solubilized in DMSO, showed a better efficiency in the classical assay with similar IC_{50} compared to the SIEFED (Figs. **4A-C**). One interpretation is that they primarily function as electron donors in the peroxidase cycle of the enzyme. However, the SIEFED assay revealed that both molecules can also inhibit the enzyme's active site, albeit with a lower IC50 for gallic acid (0.53 ± 0.04) compared to quercetin (1.37 ± 0.02). Across all plant species studied, the majority of fractions exhibited a dose-dependent inhibitory effect, with chromatographic fractions obtained using ethyl acetate (EtOAc) showing the least efficiency. (Figs. **4A-C**). Whatever the species,

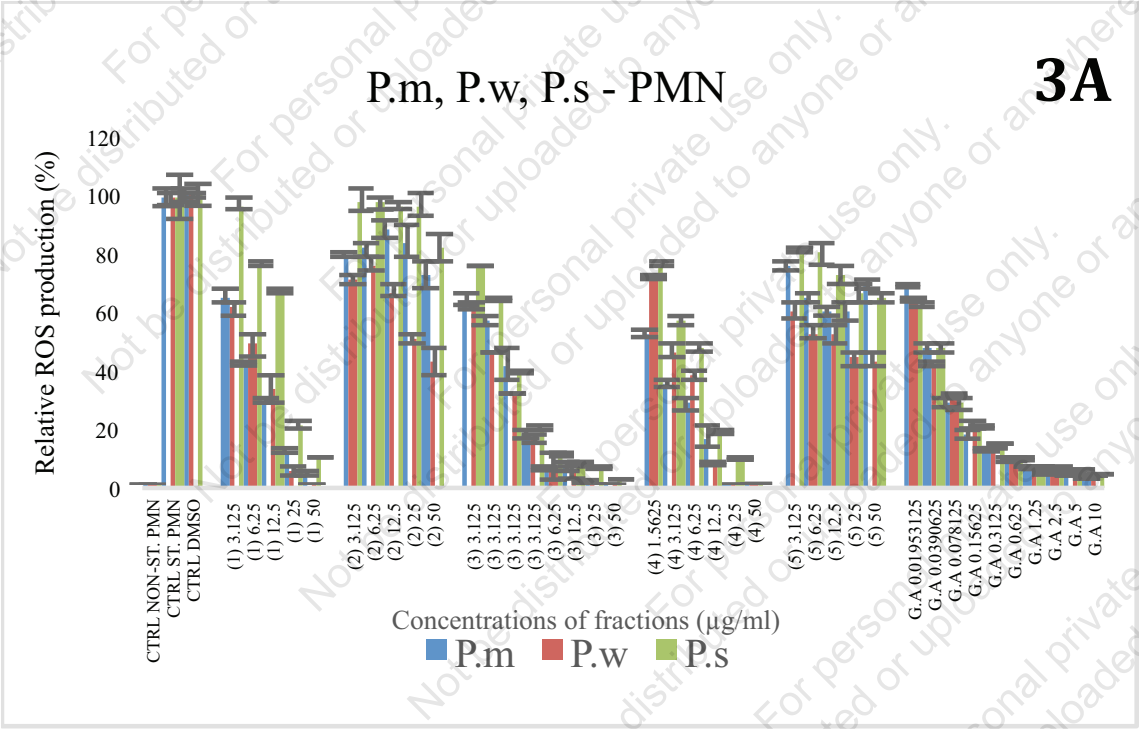

Fig. (3). Relative inhibition of neutrophils ROS production induced by *Primula macroxalyx* (P.m.) *Primula woronowii* (P.w) and *Primula saguramica* fractions obtained after ethanol extraction and freeze-drying (fraction 1) or after chromatographic separation with H_2O, MeOH (50% and 100%), and EtOAc (fractions 2, 3, 4 and 5, respectively). Gallic acid (GA) and quercetin (Qu) were used as reference compounds. All the powdered extracts were solubilized in DMSO and their ROS inhibition capacity was compared to DMSO control (Ctrl DMSO) and stimulated neutrophil without a sample (ST PMN). Results were obtained after 30 min of neutrophil stimulation with PMA and expressed in relative percentages vs ST PMN set as 100 % response (Mean +/- SD, n=3 independent experiments, with 3 technical replicates for each experiment). NON-ST PMN: Non-stimulated PMN. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

it was the chromatographic fraction obtained with 50% MeOH (3) that gave the best results, followed by the crude fraction obtained with ethanol extraction and freeze drying (1). For these two fractions, results obtained by SIEFED and classical assays were quite similar, suggesting that compounds from these fractions act mainly by blocking the active site of the enzyme. According to the IC_{50} from SIEFED and classical enzymatic assays, P.w 3 seems to have the strongest anti-MPO activity (Table **1**). For some fractions, the IC_{50} could not be determined because at these concentrations, the inhibition of MPO and electron donating ability in percentage did not exceed 50%.

4. DISCUSSION

In this article, we focused on species growing in Georgia: P.m, P.w, and P.s. (*Primula macrocalyx*, *Primula woronowii,* and *Primula saguramica*). The first step was the development of an optimal extraction condition to obtain secondary metabolites. In the literature, phenols, triterpene saponins, and flavonoids are the most common classes of compounds [59]. Based on these data, EtOH 80% was selected as an extraction solvent. Three times extraction was performed, and as a result, crude extracts were obtained (P.m1, P.w1, and P.s1). For fractionation purposes, column chromatography was carried out with Diaion HP-20 as the stationary phase and solvents with different polarities (water, MeOH 50%, MeOH 100%, and EtOAc 100%) as the mobile phase. Some compounds strengthen each other and, as a result, have synergism; however, in some cases, they interfere with other effects, so the maximum therapeutic effect is not revealed. Based on our results, extraction with highly porous solvents resulted in a high extract yield but with a low pharmacological effect as compared to non-polar ones. This indicates that fractionation has an important influence on results. In addition, the use of a combination of polar and nonpolar solvents increases the extraction efficiency of phytochemicals with good antioxidant quality from these species. After using column chromatography, the following fractions were obtained: H_2O – P.m 2, P.w 2, P.s 2, MeOH 50% - P.m 3, P.w 3, P.s 3, MeOH 100% - P.m 4, P.w 4, P.s 4, EtOAc 100% - P.m 5, P.w 5, P.s 5. For the preliminary screening of phytochemicals, several tests and TLC were performed, which confirmed the content of flavonoids and triterpene saponins. According to TLC, flavonoids are mostly in 3 fractions (P.m 3, P.w 3, P.s 3), while triterpene saponins are in 1 and 4. For flavonoids, 1% 2-aminoethyl diphenylborinate and triterpene saponins, 1% vanillin solution in methanol were used, and yellow-orange and violet spots were produced, respectively [46]. Based on the characteristics of secondary metabolites, various techniques have been employed to assess the antioxidant properties of the fractions *in vitro*. These methods involve chemical evaluations to measure the fractions' ability to neutralize radical species generated in cell-free systems (such as DPPH and ABTS assays) [60]. The ABTS*+ and DPPH* assays are effective and complementary techniques for assessing the overall stoichiometric activities of molecules. The ABTS*+ assay focuses on hydrogen atom transfer

Table 1. Determination of IC_{50} (µg/ml).

Name	ABTS (µg/ml)	DPPH (µg/ml)	SIEFED (µg/ml)	Classical (µg/ml)	PMN (µg/ml)
P.m 1	> 50	> 50	11.89 ± 0.6	9.8 ± 0.1	5.1 ± 0.3
P.w 1	45.4 ± 2.9	> 50	4.8 ± 0.4	5.9 ± 0.7	6.44 ± 0.2
P.s 1	> 50	> 50	11.58 ± 0.7	14.1 ± 0.1	16.8 ±0.02
P.m 2	> 50	> 50	17.28 ± 0.8	11.9 ± 0.1	> 50
P.w 2	> 50	> 50	5.2 ± 0.3	17.8 ± 0.1	25.4 ± 0.01
P.s 2	> 50	> 50	22.32 ± 1.02	9.25 ± 0.04	> 50
P.m 3	12.88	36.47 ± 0.13	4.44 ± 0.4	1.96 ± 0.2	1.17 ± 0.07
P.w 3	8.51 ± 0.18	34.57 ± 0.47	1.49 ± 0.7	1.89 ± 0.01	0.68 ± 0.04
P.s 3	27.9	48.88 ± 0.5	4.89 ± 0.9	2.71 ± 0.9	1.1 ± 0.03
P.m 4	> 50	> 50	> 50	10.51 ± 0.1	1.4 ± 0.03
P.w 4	48.1 ± 2.1	> 50	6.02 ± 0.2	11.53 ± 0.3	2.8 ± 0.2
P.s 4	> 50	> 50	33.9 ± 0.9	15.68 ± 0.1	5.3 ± 0.1
P.m 5	> 50	> 50	> 50	> 50	> 50
P.w 5	> 50	> 50	> 50	> 50	> 50
P.s 5	> 50	> 50	> 50	> 50	> 50
Gallic acid	0.66 ± 0.15	1.09 ± 0.02	0.53 ± 0.04	0.18 ± 0.02	0.03 ± 0.01
Quercetin	2.07 ± 0.01	2.59 ± 0.07	1.37 ± 0.02	0.18 ± 0.02	0.14 ± 0.01

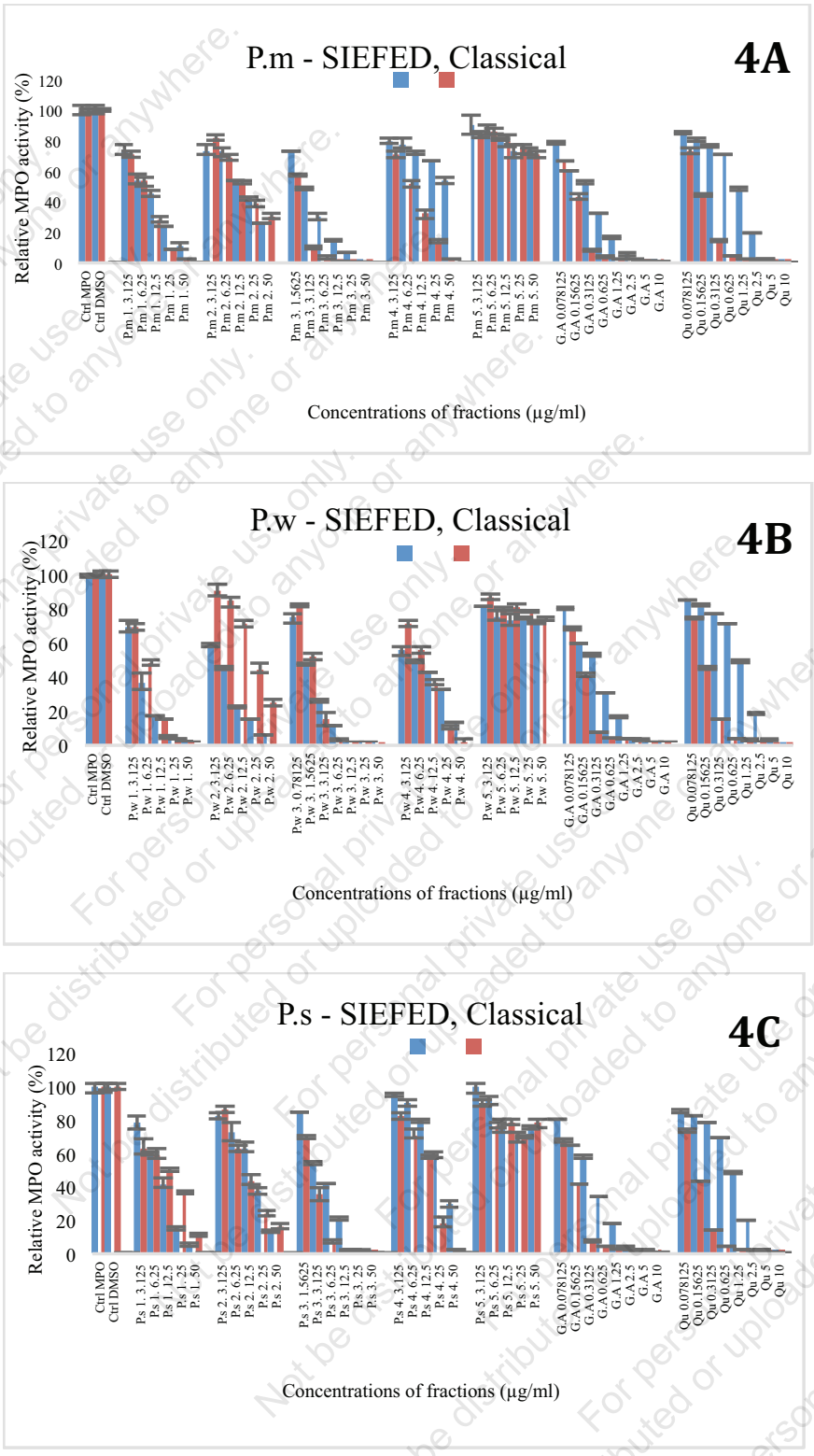

Fig. (4). Inhibition of MPO activity measured by SIEFED and classical assay by *Primula macroxalyx* (P.m.) (**A**) *Primula woronowii* (P.w) (**B**) and *Primula saguramica* (**C**) fractions obtained after ethanol extraction and freeze-drying (1) or after chromatographic separation with H_2O, 50 % MeOH, 100% MeOH, and EtOAc (fractions 2, 3, 4 and 5, respectively). Gallic acid (GA) and quercetin (Qu) were used as reference compounds. All the powdered extracts were solubilized in DMSO and their MPO inhibition was compared to DMSO control (Ctrl DMSO) and MPO control without a sample. Results were expressed in relative percentages *vs* Ctrl MPO set as 100 % (Mean +/- SD, n=3 independent experiments with 3 technical replicates for each experiment). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(HAT), where antioxidants donate hydrogen atoms. In contrast, the DPPH* assay involves both HAT and single electron transfer (SET) mechanisms. The latter reaction is based on single-electron transfer from antioxidants to free radicals [61]. From ABTS and DPPH results, the 3 species showed stoichiometric antioxidant properties when the plant fraction was prepared in 50% MEOH. According to the results, P.w showed the best antiradical activity but with a weaker response for DPPH than for ABTS. According to the TLC results, P.w3 fractions are characterized by more types of flavonoids, and this may be a reason for better antioxidant activity than others. Strong stoichiometric antioxidant properties were already demonstrated in different Primula species (*Primula veris, Primula heterochroma Primula vulga ris),* indicating that these plants are important sources of antioxidants [62, 63]. Nevertheless, the ABTS and DPPH assays do not account for potential effects on cells. Antioxidant molecules also exert their effects by inhibiting enzymes and pathways involved in ROS production (anticatalytic activity). It is apparent that anticatalytic activity can be more potent than stoichiometric activity because even at low concentrations, anticatalytic antioxidants can effectively block or reduce ROS production sources [48]. Currently, there is increasing research focusing on how molecules' antioxidant effects influence the activity of enzymes involved in oxidation processes and the formation of radical species. This requires the use of enzymatic systems and both extracellular and intracellular cellular models [64-66]. We investigated the impact of crude extract and fractions on neutrophils and MPO, both pivotal in inflammation. To assess ROS production by stimulated neutrophils, we employed the luminescent probe L012, which detects primarily superoxide anion (O2.-) generated *via* NADPH oxidase activity [67]. Our findings demonstrated significant inhibition of ROS production by neutrophils with the 50% MeOH preparations of the three Primula species (Fig. **3** and Supplementary Figs. **2A** and **B**), confirming our earlier results on their antioxidant stoichiometric activities. These 3 species are well used in Georgia for their anti-inflammatory properties, especially against cough, catarrhs of the respiratory tract, and chronic bronchitis. Neutrophils are known to be involved in common respiratory diseases. Stimulation of neutrophils and ROS production also determine the NET formation consisting of decondensed chromatin fibers coated with antimicrobial proteins, such as histones, neutrophil elastase (NE), α-defensins, and MPO [68]. Generally, excessive NET production increases mucus viscosity, causing the lungs to fill with mucus and affecting lung function. It seems that secondary metabolites contained in primula species can inhibit NET formation [69]. In our cellular model, we primarily measure superoxide anion, but we acknowledge the possibility of interactions with cells that could potentially modify cellular metabolism or enzyme activities. Flavonoids are known to exert anti-inflammatory effects through various cellular mechanisms, including modulation of signaling pathways, production of cytokines and chemokines, and regulation of enzymes involved in reactive oxygen and nitrogen species [70-72]. One such enzyme, myeloperoxidase (MPO), released by stimulated PMNs, plays a significant role in inflammation. Following its oxidant activity, which involves both peroxidase and chlorination cycles, MPO can generate potent oxidant species like HOCl [73]. Compounds generated from MPO activity are essential for the bactericidal function of neutrophils against pathogens, yet they also contribute to tissue damage and the initiation and progression of acute and chronic inflammatory conditions [74]. The SIEFED (specific immunological extraction followed by enzymatic detection) method, developed by Franck *et al*. [58, 75], enables the direct study of interactions between MPO and molecules extracted from samples. This technique involves extracting MPO using immobilized anti-MPO antibodies, followed by a washing step to remove proteins, interfering molecules, or test samples/drugs. Subsequently, MPO activity is measured using a detection system containing a fluorogenic substrate, hydrogen peroxide, and sodium nitrite as a reaction enhancer (Franck *et al*., 2005). Persistent inhibition of enzyme activity following the molecule's removal suggests that the molecule has interacted with the enzyme, possibly altering its structure and inhibiting its active site. This interference can restrict or prevent substrate access to the site [75]. In contrast, classical enzymatic assays that do not involve immunological capture allow for the evaluation of the electron donor potential of tested molecules in the MPO peroxidase cycle [76]. However, in these assays, there is a risk of interactions occurring between the substrate or products derived from enzyme activity and the inhibitor. However, it is a good technique to determine the electron-donating potential of the molecule for the peroxidase cycle of the enzyme or competitive inhibition toward the substrate. For both techniques, the 3 plant species showed good potential to inhibit MPO activity by either interacting with its active site or by substrate competition, especially the crude extract and the fraction prepared with 50% MEOH. Therefore, Primula species contained molecules having the potential to modulate the activity of MPO and thus prevent the harmful effect of the oxidant molecules derived from its activity. Flavonoid compounds are known to inhibit the activity of MPO [15]. Further studies are needed to determine which molecules from the extract are responsible for this inhibition.

CONCLUSION

Triterpene saponins and flavonoids were identified in *Primula macrocalyx*, *Primula woronowii*, and *Primula saguramica*. The antioxidant and anti-inflammatory activities of the fractions derived from these species were evaluated through a range of analytical methods. The results from all employed methods indicate that the P.w 3 fraction exhibits both significant stoichiometric and anticatalytic activity. Specifically, secondary metabolites in the P.w 3 fraction demonstrate the capacity to reduce ABTS and DPPH radicals. The SIEFED assay results show that this fraction inhibits the active site of myeloperoxidase (MPO), functions as an electron donor in the MPO peroxidase cycle, and inhibits reactive oxygen species, as evidenced by the classical enzymatic assays and experiments conducted on neutrophils. These findings may be partially attributable to the established anti-inflammatory and therapeutic properties associated with *Primula* species.

AUTHORS' CONTRIBUTIONS

L. M. and K. M. - the research design, analysis and interpretation of results; T. F. and A. M-M. M.F – supervision;

designed and performed the experiments, derived the models, as well as the data analysis and manuscript writing; C.S. and D. S. - assisted with carrying out the experiments, All authors reviewed the results and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

COX	=	Cyclo-oxygenase
NE	=	Neutrophil Elastase
PMNs	=	Polymorphonuclear Neutrophils
ROS	=	Reactive Oxygen Species
SD	=	Standard Deviation
TLC	=	Thin-layer Chromatography
XO	=	Xanthine Oxidase

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

RESEARCH INVOLVING PLANTS

The plant source used is seasonally abundant and used for the study. The authors declare that the plant species used in the study was not endangered.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analysed during this study are included in this published article and its Supplementary material.

FUNDING

This work was carried out within Erasmus + 2017 - KA107 funding program.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Barkat, M.A.; Goyal, A.; Barkat, H.A.; Salauddin, M.; Pottoo, F.H.; Anwer, E.T. Herbal Medicine: Clinical Perspective and Regulatory Status. *Comb. Chem. High Throughput Screen.,* **2021**, *24*(10), 1573-1582. http://dx.doi.org/10.2174/1386207323999201110192942 PMID: 33176638

[2] Patarkalashvili, T. Forest biodiversity of Georgia and endangered plant species. *Annals Agrarian Sci.,* **2017**, *17*(3), 349-351. http://dx.doi.org/10.1016/j.aasci.2017.06.002

[3] The World Flora Online (WFO). Primulaceae Batsch ex Borkh. **1797**. Available From: http://www.worldfloraonline.org/taxon/wfo-7000000497

[4] Mskhiladze, L. Microstructural Features of Primula woronowii Losinsk., Primula macrocalyx Bunge. and Primula saguramica Gavr. from Georgian Flora. **2023**. Available From: https://www.researchhub.com/paper/4309210/microstructural-features-of-primula-woronowii-losinsk-primula-macrocalyx-bunge-and-primula-saguramica-gavr-from-georgian-flora/conversation

[5] Alam, F.; Din, K.M.; Sarfraz, M.; Qudoos, A.; Malik, S. Genus Primula and its role in phytomedicine; a systematic review. *Phytomedicine Plus,* **2024**, *4*(1), 100510. http://dx.doi.org/10.1016/j.phyplu.2023.100510

[6] Colombo, P.S.; Flamini, G.; Rodondi, G.; Giuliani, C.; Santagostini, L.; Fico, G. Phytochemistry of European Primula species. *Phytochemistry,* **2017**, *143*, 132-144. http://dx.doi.org/10.1016/j.phytochem.2017.07.005 PMID: 28806605

[7] Li, X. Unusual Flavones from Primula macrocalyx as Inhibitors of OAT1 and OAT3 and as Antifungal Agents against Candida rugosa. *Sci Rep.,* **2019**, *9*(1), 9230.

[8] Budzianowski, J.; Wollenweber, E. Rare flavones from the glandular leaf exudate of the Oxlip, Primula elatior L. *Nat. Prod. Commun.,* **2007**, *2*(3), 1934578X0700200. http://dx.doi.org/10.1177/1934578X0700200308

[9] EMA. Assessment report on Primula veris L. and/or Primula elatior (L.) Hill, flos. **2012**. Available From: https://www.pharmacompass.com/pAssets/pdf/pubchem/primula-veris-l.pdf

[10] EMA. Assessment report on Primulaveris L. and/or Primulaelatior (L.) Hill, radix. **2012**.

[11] Pietta, P.G. Flavonoids as Antioxidants. *J. Nat. Prod.,* **2000**, *63*(7), 1035-1042. http://dx.doi.org/10.1021/np9904509 PMID: 10924197

[12] Heim, K.E.; Tagliaferro, A.R.; Bobilya, D.J. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.,* **2002**, *13*(10), 572-584. http://dx.doi.org/10.1016/S0955-2863(02)00208-5 PMID: 12550068

[13] Pérez de la Lastra, J.M. Nitration of flavonoids and tocopherols as potential modulators of nitrosative stress—a study based on their conformational structures and energy content. *Stresses,* **2022**, *2*(2), 213-230. http://dx.doi.org/10.3390/stresses2020015

[14] Walker, E.H.; Pacold, M.E.; Perisic, O.; Stephens, L.; Hawkins, P.T.; Wymann, M.P.; Williams, R.L. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol. Cell,* **2000**, *6*(4), 909-919. http://dx.doi.org/10.1016/S1097-2765(05)00089-4 PMID: 11090628

[15] Shiba, Y.; Kinoshita, T.; Chuman, H.; Taketani, Y.; Takeda, E.; Kato, Y.; Naito, M.; Kawabata, K.; Ishisaka, A.; Terao, J.; Kawai, Y. Flavonoids as substrates and inhibitors of myeloperoxidase: Molecular actions of aglycone and metabolites. *Chem. Res. Toxicol.,* **2008**, *21*(8), 1600-1609. http://dx.doi.org/10.1021/tx8000835 PMID: 18620432

[16] Timilsena, Y.P.; Phosanam, A.; Stockmann, R. Perspectives on Saponins: Food Functionality and Applications. *Int. J. Mol. Sci.,* **2023**, *24*(17), 13538. http://dx.doi.org/10.3390/ijms241713538 PMID: 37686341

[17] Barbosa, A.D.P. An overview on the biological and pharmacological activities of saponins. *Int. J. Pharm. Pharm. Sci.,* **2014**, *6*, 47-50.

[18] Shi, J.; Arunasalam, K.; Yeung, D.; Kakuda, Y.; Mittal, G.; Jiang, Y. Saponins from edible legumes: Chemistry, processing, and health benefits. *J. Med. Food,* **2004**, *7*(1), 67-78. http://dx.doi.org/10.1089/109662004322984734 PMID: 15117556

[19] Shibata, S. Saponins with biological and pharmacological activity.*New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity,* **1977**, 177-196. http://dx.doi.org/10.1007/978-3-642-66682-7_7

[20] Meshram, M.A.; Bhise, U.O.; Makhal, P.N.; Kaki, V.R. Synthetically-tailored and nature-derived dual COX-2/5-LOX inhibitors: Structural aspects and SAR. *Eur. J. Med. Chem.,* **2021**, *225*, 113804. http://dx.doi.org/10.1016/j.ejmech.2021.113804 PMID: 34479036

[21] Bukvicki, D.; Kovtonyuk, N.K.; Legin, A.A.; Keppler, B.K.; Brecker, L.; Asakawa, Y.; Valant-Vetschera, K. Hunting for bisbibenzyls in *Primula veris* subsp. macrocalyx (Bunge) Lüdi: Organ-specific accumulation and cytotoxic activity. *Phytochem. Lett.,* **2021**, *44*, 90-97. http://dx.doi.org/10.1016/j.phytol.2021.06.014

[22] Dymock, W.; Warden, C.J.; Hand Hooper, D. , **1890**.

[23] Jäger, A.K.; Gauguin, B.; Adsersen, A.; Gudiksen, L. Screening of plants used in Danish folk medicine to treat epilepsy and convulsions. *J. Ethnopharmacol.,* **2006**, *105*(1-2), 294-300. http://dx.doi.org/10.1016/j.jep.2005.10.015 PMID: 16293381

[24] Saqib, N. Phytochemical studies of some Primulaceous and leguminous plants., P.hD. Thesis, University of Karachi, **1980**.

[25] Budniak, L.; Vasenda, M.; Slobodianiuk, L. Determination of flavonoids and hydroxycinnamic acids in tablets with thick extract of *Primula denticulata* SMITH. *Pharmacol OnLine,* **2021**, *2*, 1244-1253.

[26] Kahraman, C.; Sari, S.; Küpeli Akkol, E.; Tatli Cankaya, I. Bioactive saponins of Primula vulgaris Huds. promote wound healing through inhibition of collagenase and elastase enzymes: *In vivo, in vitro and in silico* evaluations. *Chem. Biodivers.,* **2022**, *19*(12), e202200582. http://dx.doi.org/10.1002/cbdv.202200582 PMID: 36310134

[27] Kosenkova, Y.S.; Polovinka, M.P.; Komarova, N.I.; Korchagina, D.V.; Kurochkina, N.Y.; Cheremushkina, V.A.; Salakhutdinov, N.F. Riccardin C, a bisbibenzyl compound from *Primula macrocalyx*. *Chem. Nat. Compd.,* **2007**, *43*(6), 712-713. http://dx.doi.org/10.1007/s10600-007-0241-8

[28] Kosenkova, Y.S.; Polovinka, M.P.; Komarova, N.I.; Korchagina, D.V.; Morozov, S.V.; Vyalkov, A.I.; Kurochkina, N.Y.; Cheremushkina, V.A.; Salakhutdinov, N.F. Fatty-acid composition and secondary metabolites from slightly polar extracts of the aerial part of *Primula macrocalyx*. *Chem. Nat. Compd.,* **2008**, *44*(5), 564-568. http://dx.doi.org/10.1007/s10600-008-9145-5

[29] Talreja, S.; Tiwari, D.S. Complete Overview On Primula Vulgaris. *Int. J. Life Sci. Pharma Res.,* **2023**, *13*, 277-286. http://dx.doi.org/10.22376/ijlpr.2023.13.6.P277-P286

[30] Pharmacology in ancient Georgia and the ways of its further development from ancient times to XX century. *Sokrat Salukvadze,* **1987**, 188.

[31] *Zaza Panaskerteli-Tsitsishvili, healing book, karabadine", publishing house*; Soviet Georgia: Tbilisi, **1978**, p. 623.

[32] Glatthaar-Saalmüller, B.; Rauchhaus, U.; Rode, S.; Haunschild, J.; Saalmüller, A. Antiviral activity *in vitro* of two preparations of the herbal medicinal product Sinupret® against viruses causing respiratory infections. *Phytomedicine,* **2011**, *19*(1), 1-7. http://dx.doi.org/10.1016/j.phymed.2011.10.010

[33] Margineanu, C.; Cucu, V.; Grecu, L.; Pârvu, C. [The anticandida action of the saponine from Primula (author's transl)]. *Planta Med.,* **1976**, *30*(1), 35-38. http://dx.doi.org/10.1055/s-0028-1097690 PMID: 785496

[34] Demir, S.; Turan, I.; Aliyazicioglu, R.; Yaman, S.O.; Aliyazicioglu, Y. *Primula vulgaris* extract induces cell cycle arrest and apoptosis in human cervix cancer cells. *J. Pharm. Anal.,* **2018**, *8*(5), 307-311. http://dx.doi.org/10.1016/j.jpha.2018.05.003

[35] Sufka, K.J.; Roach, J.T.; Chambliss, W.G., Jr; Broom, S.L.; Feltenstein, M.W.; Wyandt, C.M.; Zeng, L. Anxiolytic properties of botanical extracts in the chick social separation-stress procedure. *Psychopharmacology (Berl.),* **2001**, *153*(2), 219-224. http://dx.doi.org/10.1007/s002130000571 PMID: 11205422

[36] Brondani, D.J.; Nascimento, C.R.; de M Moreira, D.R.; Lima Leite, A.C.; de Souza, I.A.; Bieber, L.W. Synthesis and Antitumour Activity of the Primin (2-methoxy-6-n-pentyl-1,4-benzoquinone) and Analogues. *Med. Chem.,* **2007**, *3*(4), 369-372. http://dx.doi.org/10.2174/157340607781024410 PMID: 17627574

[37] Najmus-Saqib, Q.; Alam, F.; Ahmad, M. Antimicrobial and cytotoxicity activities of the medicinal plant *Primula macrophylla*. *J. Enzyme Inhib. Med. Chem.,* **2009**, *24*(3), 697-701. http://dx.doi.org/10.1080/14756360802333406 PMID: 18686139

[38] Bhatt, H.; Saklani, S.; Upadhayay, K. Anti-oxidant and anti-diabetic activities of ethanolic extract of *Primula denticulata* flowers. *Indones. J. Pharm.,* **2016**, *27*(2), 74. http://dx.doi.org/10.14499/indonesianjpharm27iss2pp74

[39] Ahmad, V.U.; Shah, M.G. Macrophyllicinin, a saponin from Primula macrophylla. *J. Nat. Prod.,* **1993**, *56*(9), 1580-1585. http://dx.doi.org/10.1021/np50099a018 PMID: 8254351

[40] Ruiz-López, N.; Haslam, R.P.; Venegas-Calerón, M.; Larson, T.R.; Graham, I.A.; Napier, J.A.; Sayanova, O. The synthesis and accumulation of stearidonic acid in transgenic plants: A novel source of 'heart-healthy' omega-3 fatty acids. *Plant Biotechnol. J.,* **2009**, *7*(7), 704-716. http://dx.doi.org/10.1111/j.1467-7652.2009.00436.x PMID: 19702757

[41] Mats, M.N.; Savchenko, O.N. Mechanism of the contraceptive action of the triterpene glycosides from the rock jasmine *Androsace septentrionalis* L. *Farmakol. Toksikol.,* **1986**, *49*(2), 38-39. PMID: 3086118

[42] Wekipedia. Georgian Soviet Encyclopedia. **1986**. Available From: https://en.wikipedia.org/wiki/Georgian_Soviet_Encyclopedia

[43] Burdon, R.H. *Laboratory Techniques in Biochemistry and Molecular Biology*; Elsevier: Amsterdam, **1991**.

[44] Harborne, J.B. *Phytochemical methods: A guide to modern techniques of plant analysis,* 2nd ed; Chapman and Hall: London, **1998**, pp. 54-84.

[45] Kokate, K.C. *Practical pharmacognosy,* 4th ed; Vallabh Prakashan: Delhi, **1997**, p. 218.

[46] Wagner, H. *Plant Drug Analysis, A Thin Layer Chromatography Atlas.,* **1996**,

[47] Miller, N.J.; Rice-Evans, C.; Davies, M.J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci. (Lond.),* **1993**, *84*(4), 407-412. http://dx.doi.org/10.1042/cs0840407 PMID: 8482045

[48] Franck, T.; Mouithys-Mickalad, A.; Robert, T.; Ghitti, G.; Deby-Dupont, G.; Neven, P.; Serteyn, D. Differentiation between stoichiometric and anticatalytic antioxidant properties of benzoic acid analogues: A structure/redox potential relationship study. *Chem. Biol. Interact.,* **2013**, *206*(2), 194-203. http://dx.doi.org/10.1016/j.cbi.2013.09.009 PMID: 24060682

[49] Wagner, H. Phenolic Compounds: Structure, Classification, and Antioxidant Power.*Bioactive Compounds: Health Benefits and Potential Applications*; Woodhead Publishing: Sawston, United Kingdom, **2019**. http://dx.doi.org/10.1016/B978-0-12-814774-0.00002-5

[50] Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature,* **1958**, *181*(4617), 1199-1200. http://dx.doi.org/10.1038/1811199a0

[51] Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and mechanisms of antioxidant activity using the DPPH• free radical method. *Lebensm. Wiss. Technol.,* **1997**, *30*(6), 609-615. http://dx.doi.org/10.1006/fstl.1997.0240

[52] Etsè, K.S.; Etsè, K.D.; Nyssen, P.; Mouithys-Mickalad, A. Assessment of anti-inflammatory-like, antioxidant activities and molecular docking of three alkynyl-substituted 3-ylidene-dihydrobenzo[d]isothiazole 1,1-dioxide derivatives. *Chem. Biol. Interact.,* **2021**, *344*, 109513. http://dx.doi.org/10.1016/j.cbi.2021.109513 PMID: 33974901

[53] Benbarek, H.; Deby-Dupont, G.; Deby, C.; Caudron, I.; Mathy-Hartert, M.; Lamy, M.; Serteyn, D. Experimental model for the study by chemiluminescence of the activation of isolated equine leucocytes. *Res. Vet. Sci.,* **1996**, *61*(1), 59-64. http://dx.doi.org/10.1016/S0034-5288(96)90112-5 PMID: 8819196

[54] Kim, J.A.; Neupane, G.P.; Lee, E.S.; Jeong, B.S.; Park, B.C.; Thapa, P. NADPH oxidase inhibitors: A patent review. *Expert Opin Ther Pat.,* **2011**, *21*(8), 1147-58.

[55] Goel, G.; Makkar, H.P.S.; Francis, G.; Becker, K. Phorbol esters: Structure, biological activity, and toxicity in animals. *Int. J. Toxicol.,* **2007**, *26*(4), 279-288. http://dx.doi.org/10.1080/10915810701464641 PMID: 17661218

[56] Pagani, A.; Gaeta, S.; Savchenko, A.I.; Williams, C.M.; Appendino, G. An improved preparation of phorbol from croton oil. *Beilstein J. Org. Chem.*, **2017**, *13*, 1361-1367. http://dx.doi.org/10.3762/bjoc.13.133 PMID: 28781702

[57] Franck, T.; Kohnen, S.; de la Rebière, G.; Deby-Dupont, G.; Deby, C.; Niesten, A.; Serteyn, D. Activation of equine neutrophils by phorbol myristate acetate or N-formyl-methionyl-leucyl-phenylalanine induces a different response in reactive oxygen species production and release of active myeloperoxidase. *Vet. Immunol. Immunopathol.,* **2009**, *130*(3-4), 243-250. http://dx.doi.org/10.1016/j.vetimm.2009.02.015 PMID: 19328559

[58] Franck, T.; Kohnen, S.; Deby-Dupont, G.; Grulke, S.; Deby, C.; Serteyn, D. A specific method for measurement of equine active myeloperoxidase in biological samples and in *in vitro* tests. *J. Vet. Diagn. Invest.,* **2006**, *18*(4), 326-334. http://dx.doi.org/10.1177/104063870601800402 PMID: 16921870

[59] Aslam, K.; Nawchoo, I.A.; Bhat, M.A.; Ganie, A.H.; Aslam, N. Ethno-pharmacological review of genus Primula. *Int. J. Adv. Res. (Indore),* **2014**, *2*(4), 29-34.

[60] Arteaga, J.F.; Ruiz-Montoya, M.; Palma, A.; Alonso-Garrido, G.; Pintado, S.; Rodríguez-Mellado, J.M. Comparison of the simple cyclic voltammetry (CV) and DPPH assays for the determination of antioxidant capacity of active principles. *Molecules,* **2012**, *17*(5), 5126-5138. http://dx.doi.org/10.3390/molecules17055126 PMID: 22555300

[61] Liang, N.; Kitts, D. Antioxidant property of coffee components: Assessment of methods that define mechanisms of action. *Molecules,* **2014**, *19*(11), 19180-19208. http://dx.doi.org/10.3390/molecules191119180 PMID: 25415479

[62] Noroozisharaf, A.; Samizadeh Lahiji, H.; Hatamzadeh, A.; Bakhshi, D. Phytochemical attributes of endemic endangered primrose (Primula heterochroma Stapf.) accessions grown in Iran. *Physiol. Mol. Biol. Plants,* **2015**, *21*(4), 573-581. http://dx.doi.org/10.1007/s12298-015-0328-9 PMID: 26600683

[63] Tarapatskyy, M.; Gumienna, A.; Sowa, P.; Kapusta, I.; Puchalski, C. Bioactive Phenolic Compounds from *Primula veris* L.: Influence of the Extraction Conditions and Purification. *Molecules,* **2021**, *26*(4), 997. http://dx.doi.org/10.3390/molecules26040997 PMID: 33668513

[64] Derochette, S.; Franck, T.; Mouithys-Mickalad, A.; Deby-Dupont, G.; Neven, P.; Serteyn, D. Intra- and extracellular antioxidant capacities of the new water-soluble form of curcumin (NDS27) on stimulated neutrophils and HL-60 cells. *Chem. Biol. Interact.,* **2013**, *201*(1-3), 49-57. http://dx.doi.org/10.1016/j.cbi.2012.12.010 PMID: 23291280

[65] Wolfe, K.L.; Liu, R.H. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.,* **2007**, *55*(22), 8896-8907. http://dx.doi.org/10.1021/jf0715166 PMID: 17902627

[66] Malle, E.; Furtmüller, P.G.; Sattler, W.; Obinger, C. Myeloperoxidase: A target for new drug development? *Br. J. Pharmacol.,* **2007**, *152*(6), 838-854. http://dx.doi.org/10.1038/sj.bjp.0707358 PMID: 17592500

[67] Li, Y.; Zhu, H.; Kuppusamy, P.; Roubaud, V.; Zweier, J.L.; Trush, M.A. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J. Biol. Chem.,* **1998**, *273*(4), 2015-2023. http://dx.doi.org/10.1074/jbc.273.4.2015 PMID: 9442038

[68] Porto, B.N.; Stein, R.T. Neutrophil Extracellular Traps in Pulmonary Diseases: Too Much of a Good Thing? *Front. Immunol.,* **2016**, *7*, 311. http://dx.doi.org/10.3389/fimmu.2016.00311 PMID: 27574522

[69] Seibel, J.; Wonnemann, M.; Werz, O.; Lehner, M.D. A tiered approach to investigate the mechanism of anti-inflammatory activity of an herbal medicinal product containing a fixed combination of thyme herb and primula root extracts. *Clin. Phytosci.,* **2018**, *4*(1), 4. http://dx.doi.org/10.1186/s40816-018-0062-2

[70] González, R.; Ballester, I.; López-Posadas, R.; Suárez, M.D.; Zarzuelo, A.; Martínez-Augustin, O.; Medina, F.S.D. Effects of flavonoids and other polyphenols on inflammation. *Crit. Rev. Food Sci. Nutr.,* **2011**, *51*(4), 331-362. http://dx.doi.org/10.1080/10408390903584094 PMID: 21432698

[71] Ciz, M.; Denev, P.; Kratchanova, M.; Vasicek, O.; Ambrozova, G.; Lojek, A. Flavonoids inhibit the respiratory burst of neutrophils in mammals. *Oxid. Med. Cell. Longev.,* **2012**, *2012*, 1-6. http://dx.doi.org/10.1155/2012/181295 PMID: 22577489

[72] Ribeiro, D.; Freitas, M.; Lima, J.L.C.; Fernandes, E. Flavonoids inhibit the production of cytokines/chemokines and induce apoptosis in human neutrophils. *Free Radic. Biol. Med.,* **2014**, *75*(Suppl. 1), S46. http://dx.doi.org/10.1016/j.freeradbiomed.2014.10.806 PMID: 26461385

[73] Davies, M.J. Myeloperoxidase-derived oxidation: Mechanisms of biological damage and its prevention. *J. Clin. Biochem. Nutr.,* **2010**, *48*(1), 8-19. http://dx.doi.org/10.3164/jcbn.11-006FR PMID: 21297906

[74] Delporte, C.; Franck, T.; Noyon, C.; Dufour, D.; Rousseau, A.; Madhoun, P.; Desmet, J.M.; Serteyn, D.; Raes, M.; Nortier, J.; Vanhaeverbeek, M.; Moguilevsky, N.; Nève, J.; Vanhamme, L.; Van Antwerpen, P.; Zouaoui Boudjeltia, K. Simultaneous measurement of protein-bound 3-chlorotyrosine and homocitrulline by LC–MS/MS after hydrolysis assisted by microwave: Application to the study of myeloperoxidase activity during hemodialysis. *Talanta,* **2012**, *99*, 603-609. http://dx.doi.org/10.1016/j.talanta.2012.06.044 PMID: 22967600

[75] Franck, T.; Kohnen, S.; Boudjeltia, K.Z.; Van Antwerpen, P.; Bosseloir, A.; Niesten, A.; Gach, O.; Nys, M.; Deby-Dupont, G.; Serteyn, D. A new easy method for specific measurement of active myeloperoxidase in human biological fluids and tissue extracts. *Talanta,* **2009**, *80*(2), 723-729. http://dx.doi.org/10.1016/j.talanta.2009.07.052 PMID: 19836543

[76] Nyssen, P.; Franck, T.; Serteyn, D.; Mouithys-Mickalad, A.; Hoebeke, M. Propofol metabolites and derivatives inhibit the oxidant activities of neutrophils and myeloperoxidase. *Free Radic. Biol. Med.,* **2022**, *191*, 164-175. http://dx.doi.org/10.1016/j.freeradbiomed.2022.08.039 PMID: 36064069