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Phytochemical and pharmacological characterization of primula species from Georgian Flora

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Abstract

Although primula species are abundant in phytochemicals, little is known about their pharmacological characteristics, especially those of endemic species. The purpose of this study was to examine the biological activity and phytochemical composition of the Georgian endemic *Primula saguramica,*as well as *Primula macrocalyx*, and the Caucasian endemic *Primula woronowii*. Folin–Ciocalteu and aluminum chloride colorimetric assays were used to measure the total phenolic content (TPC) and total flavonoid content (TFC), respectively. Cytotoxicity was assessed using the MTT assay in RAW 264.7 murine macrophages (ATCC-TIB-71), A549 lung adenocarcinoma cell line (ATCC-CCL-185), U87MG human glioblastoma cell line (ATCC-HTB-14), and NIH/3T3 - murine fibrobalst cell line (ATCC-CRL-1658), in addition to the hemolysis assay on human erythrocytes. The ABTS radical scavenging assay was used to confirm the antioxidant activity. An individual compound from the most active fraction (P.w.3, 50% methanolic fraction of *P. woronowii*) was isolated and identified using preparative high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). For the quantitative determination of the individual compound in crude extracts and fractions, HPLC analysis was carried out, and its predicted pharmacological profile was evaluated using an *in silico* approach. P.w.3 had the highest TPC and TFC content (41.84 ± 0.08 mg GAE/g and 36.02 ± 0.2 mg QE/g) among all studied samples. It did not exhibit cytotoxic or hemolytic activity; however, it revealed potent antioxidant capacity (IC_{50} = 7.35 ± 0.23 µg/mL), which was significantly stronger than clitorin (13.25 ± 0.24 µg/mL) and the crude extract P.w.1 (42.01 ± 0.21 µg/mL). A flavonoid glycoside, clitorin (kaempferol 3-O-(2'',6''-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside), was identified from P.w.3 Clitorin content in P.w.3 was approximately ~5-fold higher (16.93 ± 1.16%, RSD) compared with P.w.1 (2.86 ± 2.5%, RSD). An *in silico* analysis showed that clitorin may have multifunctional biological activities, such as antioxidant, anti-inflammatory, antiproliferative, and vasoprotective effects. P.w.3 contained abundant bioactive flavonoids and exhibited strong antioxidant properties, without displaying cytotoxic or hemolytic effects. The isolated compound, clitorin, may serve as a lead in the discovery of phytotherapeutics for oxidative stress associated diseases.

Keywords: *Primula woronowii*; *Primula macrocalyx*; *Primula saguramica*; Clitorin; Cytotoxicity; Antioxidant; Hemolysis; In silico modeling

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1. Introduction

Worldwide distributed Primula species are characterized by notable phytochemical diversity [1-3]. Flavonoids are a well-known class of polyphenolic compounds with powerful antioxidant, anti-inflammatory, anticancer, antimicrobial, and neuroprotective activities [4–9]. The bioactivity of flavonoids is highly related to their structural properties, including the number and position of hydroxyl groups, the existence of a C2-C3 double bond, and a 4-oxo group, which overall increase their free radical scavenging and metal-chelating ability [8]. Moreover, their ability to donate hydrogen atoms or electrons enables direct neutralization of ROS and RNS, while their metal-chelating properties prevent the Fenton reaction and subsequent hydroxyl radical formation [10]. These compounds also modulate important intracellular signaling pathways such as NF-κB and MAPK, and inhibit pro-inflammatory enzymes such as COX, LOX, and xanthine oxidase. Flavonoids further activate the Nrf2/ARE pathway, leading to the upregulation of phase II detoxifying and antioxidant enzymes (SOD, CAT, GPx), thereby strengthening endogenous defense systems [10]. Flavonoids glycosylation frequently enhances solubility and stability values without a decline in bioactivity [11].

Triterpene saponins, another major class of Primula secondary metabolites, exhibit wide pharmacological properties, for instance, anti-inflammatory, immunomodulatory, antimicrobial, hepatoprotective, and cytotoxic effects [12–14]. Their amphiphilic character allows interaction with biological membranes and, in particular, disruption of cholesterol-rich membranes, which represents a source of hemolytic, membrane-permeabilizing, as well as antimicrobial and adjuvant activities [13]. They also affect immune responses by stimulating antigen-presenting cells and enhancing both humoral and cellular immunity, which explains their use as vaccine adjuvants [15]. Triterpene saponins have been demonstrated to induce apoptosis, inhibit the angiogenesis and suppress the proliferation of tumor cells by the inhibiting NF-κB, PI3K/Akt and other vital pathways in cancer models [14-16]. Additionally, the results of the phytochemical methods (TLC) indicate that flavonoids are mainly present in the 50% methanolic fractions, while the triterpene saponins are mainly concentrated in the 100% methanolic fractions of *P. macrocalyx* (P.m.*), P. woronowii* (P.w.), and *P. saguramica* (P.s.). This chemical partitioning is related to the different biological properties of these fractions [7].

It has to be emphasized that the bioactivities of flavonoids and triterpenes intersect through common molecular targets, namely the modulation of reactive oxygen species (ROS), which are important signaling molecules under physiological conditions and mediators of oxidative stress when overproduced. Flavonoids can both directly scavenge ROS and indirectly elevate cellular antioxidant defenses through activation of the Nrf2/ARE pathway, whereas perturbation of ROS levels by triterpenes can either promote apoptosis in cancer cells or, at lower doses, trigger adaptive antioxidant responses — this dose- and context-dependence is important when interpreting *in vitro* antioxidant assays [17]. Increased ROS levels are associated with many human diseases such as chronic inflammation and cancer [9–12]. Elevated ROS in tumor cells contribute to malignant progression, as well as metabolic derangements and genetic mutations leading to therapy resistance. In addition, it is known that ROS-induced activation of transcription factors including NF-κB also contributes to tumor growth by triggering survival, angiogenesis, and inflammation [13-18]. Metabolism of AA (arachidonic acid) to pro-inflammatory mediators through enzymatic pathways that utilize COX, LOX and cytochrome P450 can also be used to connect inflammation with carcinogenesis. COX-2 in particular is up-regulated in such malignancies and constitutes a key component of the tumor microenvironment that fuels inflammatory responses promoting cancer progression [19,20].

In light of the above, the determination of the cytotoxicity of triterpene-rich fractions and the antioxidant and anti-inflammatory properties of flavonoid rich fractions is important in assessing their therapeutic significance. Triterpene saponins are also reported to interact with membrane sterols [21], so their hemolysis would also have to be evaluated in order to obtain safety profiles for eventual industrial or pharmaceutical use.

Taking all these considerations into account, it highlights the necessity for a holistic phytochemical and pharmacological screening of Primula species, in terms of comparing the crude extracts and their related solvent-partitioned fractions. Beyond in vitro assays, pharmacokinetic profiling (metabolism, conjugation to glucuronides/sulfates, plasma stability), in vivo efficacy/safety, and structure–activity relationship studies will be essential to translate promising fractions into lead compounds — and testing for synergistic or antagonistic interactions between flavonoid- and saponin-rich fractions can reveal whether combinations improve therapeutic windows or raise toxicity concerns [17]. These studies are important for the discovery of safe, bioactive compounds that could potentially be used to help control oxidative stress, inflammatory disease and cancer.

2. Material and methods

2.1. Plant material

Plant materials of Primula species were collected from different locations in Georgia [2]. The dried, powder-grinded aerial parts were extracted with 80% ethanol, and the resulting crude extracts were coded as P.m.1, P.w.1 and P.s.1. Further purification was performed by column chromatography on Diaion HP-20 resin as the stationary phase. Elution was carried out stepwise using water (100%), a 1:1 water/methanol mixture, and pure methanol (100%). At the end of the process, different fractions were obtained: fractions in water (P.m.2, P.w.2, P.s.2), 50 % methanolic fractions (P.m.3, P.w.3, P.s.3) and 100% methanolic fractions (P.m.4, P.w.4, P.s.4) [23].

2.2. Chemicals and Reagents

PBS, RPMI, DMEM were obtained from GIBCO® (Thermo Fisher Scientific, Massachusetts, USA), RAW 264.7, A549, U87MG, 3T3, MTT, Triton X-100, Trypsin, 10% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate, isopropyl, 0.04N HCL, 0.4% methyl blue, methanol, deuterated methanol, dimethylsulpoxide, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride, sodium acetate were obtained from Merck & Co., Inc., Rahway, NJ, USA.

2.3. Cell lines

The following cell lines were used in this study: RAW 264.7, a murine macrophage cell line originally derived from a tumor induced by the Abelson murine leukemia virus in a male mouse; A549, a widely used human non-small cell lung carcinoma cell line; U87MG, a glioblastoma cell line isolated from a male patient with malignant glioma; and NIH/3T3, a fibroblast cell line established from an NIH/Swiss mouse embryo. All cell lines were seeded at a density of 6×10^4 cells per well. Before conducting the experiments, the cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin-glutamine (PSG). To sustain appropriate cell growth, the medium was refreshed every 2-3 days. All cultures were kept at 37^0C in a humidified chamber with 5% CO_2.

2.4. MTT assay

Cell viability was assessed by MTT assay as described previously [24,25]. Briefly, cells were seeded at a density of 6 x 10^4 in 96-well culture microplates. Subsequently, crude extracts and fractions (6.25–500 µg/mL) were added to the cultures and incubated for 24h at 37^0C. After treatment, 10 µL of MTT solution was introduced into each well, followed by a 4h incubation with 5% CO_2, at 37^0C. The resulting formazan crystals were dissolved in isopropanol containing 0.04 N HCl, and absorbance was recorded at 570nm using a spectrophotometer [20]. All experiments were carried out in triplicate. Cell viability was expressed as a percentage of the control using the equation: Cell viability (% of control) = (Absorbance of sample / Absorbance of control) x 100

2.5. Hemolysis assay

Hemolytic activity of the P.m.3, P.w.3 and P.s.3 fractions were determined as described in a previous publication [26]. Human blood samples from five healthy volunteers were provided by the Organ and Blood Donation Agency (ADOS; Santiago de Compostela. Spain) after being informed and written consent was obtained and with the approval of the Institutional Ethics Committee for Research: Comité Ético de Investigación de Galicia (CEIC; # 2014/543). 1mL of red blood cell (RBC) pellet was transferred to test tubes, adjusted to a 10 mL volume with sterile PBS and centrifuged at 400 g for 5 min. The RBC pellet was suspended in PBS and plated on 96-wells plates. It was incubated at 37°C for 4 and 24 h with the fractions. Positive and negative controls consisted of Triton X-100 (1% v/v) and PBS, respectively. The absorbance of hemoglobin in the supernatant was read at 570 nm with a microplate reader (Synergy H1 Hybrid Multi-Mode, BioTek, Winooski, US). Hemolysis percentage was calculated according to the formula: % hemolysis = (AS–ANC)/(APC–ANC) × 100, where AS, ANC and APC indicate the absorbances of the sample, negative control and positive control, respectively [26]. All measurements were carried out in triplicate.

2.6. Isolation and Structural Elucidation of the Compound Using Preparative HPLC and NMR

The major bioactive component of the P.w.3 fraction was isolated by preparative HPLC. This approach was chosen due to having high resolution, flexibility, and efficient to purify target compounds from complex plants matrices. The 30% methanol phase was used as mobile phase as optimized initially. The P.w.3 fraction was dissolved in 50% methanol to reach a concentration of 100 mg/mL, and used for injection.

The purified compound was structurally identified after being isolated by NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AVANCE NEO 500 MHz spectrometer with a cryogenic broadband probe (cryoprobe) cooled with liquid nitrogen for higher sensitivity. One-dimensional (1D) and two-dimensional (2D) NMR experiments were performed with standard pulse sequences. Deuterated methanol (CD_3OD) was employed as the solvent for preparing the sample. Based on NMR spectral data, the new compound was elucidated to be kaempferol 3-0- (2, 6-O-di-rhamnopyranosyl)-β-D-glucopyranoside (clitorin) and its molecular formula and weight was $C_{33}H_{40}O_{19}$ and 740.66g/mol, respectively.

2.7. In silico experiment

The possible biological activities of clitorin were assessed in in silico experiment using the PASS (prediction of activity spectra for substances) [27] and GUSAR (General Unrestricted Structure Activity Relationships) software [28]. PASS forecasts more than 3500 types of biological activity, covering over 300 pharmacological effects, mechanisms of action, toxic and adverse effects, interactions with enzymes and transporters. The prediction relies on the analysis of structure-activity relationships for over 250,000 biologically active substances by comparing the structure of compound under investigation with the structure of a well-known bioactive compound (drug, drug candidate, lead, etc.), The anticipated outcome is expressed as a probability of either biological activity (Pa) or inactivity (Pi). Pa > 0.7 indicates that the compound is likely an analogue of a known pharmacological agent and is predicted to exhibit activity in in vivo experiments. Compound having 0.5 < Pa < 0.7, is expected to show activity in an experiment with lower likelihood, and it differs from recognized drugs. If Pa is less than 0.5, the compound will not be expected to exhibit any activity. The GUSAR (General Unrestricted Structure–Activity Relationships) is a tool to predict the LD_{50} in rodents for various administration routes. The tool uses a database of about 10,000 chemical compounds.

2.8. HPLC-Based Quantitative Analysis of the Isolated Compound

For the validation and quantitative analysis, a stock solution of clitorin was prepared at a concentration of 1 mg/mL in 100% methanol. This solution was used to obtain a series of clitorin solutions at different concentrations (n = 5). The series of solutions was prepared on three different days. The concentration range of clitorin was 0.0625–1.0 mg/mL. The standard solutions were filtered through a 0.45 µm membrane filter. The concentration of clitorin was determined in the crude extract and fractions of *P. woronowii*, each prepared at a concentration of 10 mg/mL.

Table 1 Chromatographic Conditions (HPLC)

Column	**Eclipse plus C18 5µm 4.6mm*250 mm**
Column Temperature	$25^{0}C$
Solvent System	Acetonitrile (B) and water acidified with 0.1% formic acid (A)
Volume of the analytical solution	10 µL
Flow	0.8 mL/min
Wavelength	254 nm
Run Time	23 min

Table 2 Gradient elution program for the HPLC analysis.

Time	**A (%)**	**B (%)**	**Flow**
0.00	80	20	0.8 mL/min
15.00	70	30	0.8 mL/min
15.01	0	100	0.8 mL/min
20.00	0	100	0.8 mL/min
20.01	80	20	0.8 mL/min

The linearity of the high-performance liquid chromatography (HPLC) method was determined for clitorin. Calibration curves were constructed based on the relationship between the concentration of the test solutions and the peak area. The correlation coefficient (R^2) was determined (Fig.1), as well as the parameters for the limit of detection (LOD) and the limit of quantification (LOQ) (Fig.1).

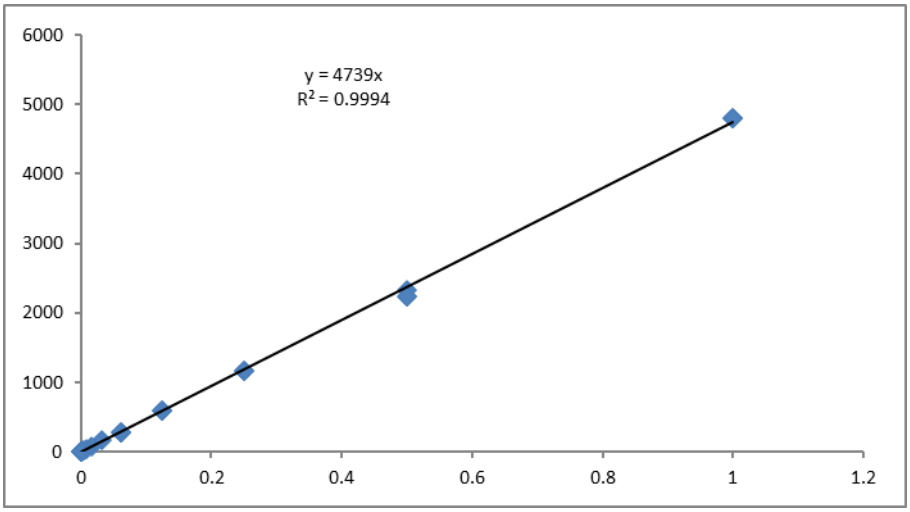

Figure 1 Calibration curve of Clitorin

2.9. ABTS Radical Scavenging Method

The slightly modified ABTS assay was used for evaluating the total antioxidant capacity of biological fluids and pharmaceutical solutions, based on the absorbance of the $ABTS^{+}$• radical cation [29].

The ABTS radical scavenging activity of each sample was determined at 760 nm using a HBS-1096 Microplate Reader. Gallic acid was used as a standard at concentrations ranging from 10 to 0.078125 µg/mL.

The samples — P.w.1, P.w.3 and clitorin — were tested at concentrations of 100–0.78125 µg/mL. Samples and standard were dissolved in DMSO and pipetted into 96-well UV microplates. The negative control was performed using DMSO (0.02 mL) with 1.98 mL of the ABTS solution. All measurements were repeated three times to provide an accurate and reliable results [30].

2.10. Quantitative Estimation of Total Phenolic Compounds

Folin–Ciocalteu (F–C) assay is a commonly used colorimetric based assay for the measurement of total phenolic content (TPC) in food, plant extracts and biological samples. The principle of F–C assay is the single electron transfer (SET) process, where phenolic compounds are the reducing components that donate their electrons to the F–C reagent [31].

The total phenolic content (TPC) of the three crude extracts (P.m.1, P.w.1 and P.s.1) as well as 50% methanolic fractions (P.m.3, P.w.3 and P.s.3) was prepared at 2 mg/mL concentration. The standard gallic acid was serially diluted (10, 20, 30, 40, 50, and 60 µg/mL). 20 µL of the sample was mixed with 20 µL of Folin–Ciocalteu reagent and incubated for 1 min. Following a 5 min rest, 200 µL of 7% Na_2CO_3 solution and 10 µL of deionized water were added, shaken again for 1 min, and incubated in the dark for 120 min at room temperature. Then, the absorbance was read at 750 nm using a microtiter plate reader. The total phenolic content of each sample was determined from the gallic acid standard curve and expressed as milligram of gallic acid equivalents per gram of dry extract (mg GAE/g) [32].

2.11. Quantitative Estimation of Total Flavonoid Compounds

The colorimetric $AlCl_3$ method is based on the formation of stable complexes between flavonoids and aluminum ions (Al^{3+}). This interaction is mainly composed of the C-4 carbonyl group with the hydroxyl group at C-3 or C-5, and of the ortho-dihydroxy groups in the A or B ring of the flavonoid structure. With Al^{3+} a yellow complex is produced, which can be measured by UV-Vis spectrophotometry, usually at 430 nm. The amount of flavonoid in the sample is directly proportional to the intensity of the yellow color [33].

For determining total flavonoid content (TFC), the crude extracts (P.m.1, P.w.1 and P.s.1) and fractions (P.m.3, P.w.3 and P.s.3) were prepared at a concentration of 2 mg/mL. Quercetin was diluted in a gradient of 40, 80, 120, 160, and 200 µg/mL. A microplate protocol-based TFC assay was performed with aluminum chloride. 50 µL of the sample extract or standard solution was added to each well, followed by 100 µL of methanol. 20 µL of 10% $AlCl_3$ was added to each well. After shaking, the mixture was incubated for 3 min, followed by the addition of 20 µL of 1 M sodium acetate and 60 µL of methanol to each well. The plate was then incubated in the dark for 40 min, and the absorbance was read at 430 nm in a HBS-1096 Microplate Reader [32].

2.12. Statistical analysis

For ABTS and cytotoxicity assays, each experiment was performed at least three times. Data are expressed as mean mean ± standard deviation (SD) relative to the control, which was set as 100%. IC_{50} values were determined by nonlinear regression using a four-parameter logistic model, with 95% confidence intervals and coefficient of determination (R^2) reported. All analyses were performed with GraphPad Prism version 10.6.0.890 (GraphPad Software, San Diego CA, USA).

3. Results and discussion

3.1. Cytotoxicity effects of extracts on different cell lines

The cytotoxicity of the crude extracts and fractions were evaluated using the MTT colorimetric assay applied to RAW 264.7, A549, U87MG, and 3T3 cell lines. A strong cytotoxic effect was observed for the 100% methanolic fractions, especially for P.m.4 and P.s.4, where IC_{50} values ranging from 20.21 to 32.66 µg/mL. On the other hand, 50% methanolic fractions (P.m.3, P.w.3, P.s.3) demonstrate no cytotoxicity at concentrations up to 500 µg/mL.

Table 3 IC_{50} (µg/ml) of crude extracts and fractions from Primula species* (Mean ± SD)

Fractions	Cell lines			
	RAW 264.7	**A549**	**U87MG**	**3T3**
P.m.1	151.5±0.25	98.84±0.08	117±0.06	111.4±0.07
P.m.2	> 500	> 500	> 500	> 500
P.m.3	> 500	> 500	> 500	> 500
P.m.4	**26.94±0.126**	**26.79±0.124**	**26.38±0.122**	**25.74±0.07**
P.w.1	98.64±0.12	193.78±0.11	115.5±0.09	116.9±0.1
P.w.2	> 500	> 500	> 500	> 500
P.w.3	> 500	> 500	> 500	> 500
P.w.4	**30.1±0.09**	**59.23±0.118**	**29.82±0.115**	**61.74±0.116**
P.s.1	126.4±0.14	248.48±0.07	128.7±0.13	123.8±0.09
P.s.2	> 500	> 500	> 500	> 500
P.s.3	> 500	> 500	> 500	> 500
P.s.4	**26.49±0.121**	**32.66±0.117**	**28.32±0.04**	**20.21±0.117**

* P.m - P. macrocalyx, P.w - P.woronowii and P.s - P.saguramica

These results are consistent with the chemical composition; 100% methanol fractions are saturated with triterpene saponins, a membrane-disruptive and pro-apoptotic agent [34], and 50% methanol fractions are enriched in flavonoids, which in general are cytoprotective. P.w.3 is non-cytotoxic, thus justifying its choice for additional studies and drug development.

3.2. Hemolytic effects of extracts on human erythrocytes

Hemolysis assays were conducted to evaluate the membrane-damaging potential of P.m.3, P.w.3, and P.s.3. None of the fractions exhibited considerable hemolytic activity, pointing to a safety factor for red blood cells. This is also true for

P.w.3 which, despite being highly enriched in bioactive flavonoids, does not show membrane-permeabilizing effects observed in saponin rich fractions (Fig. 2).

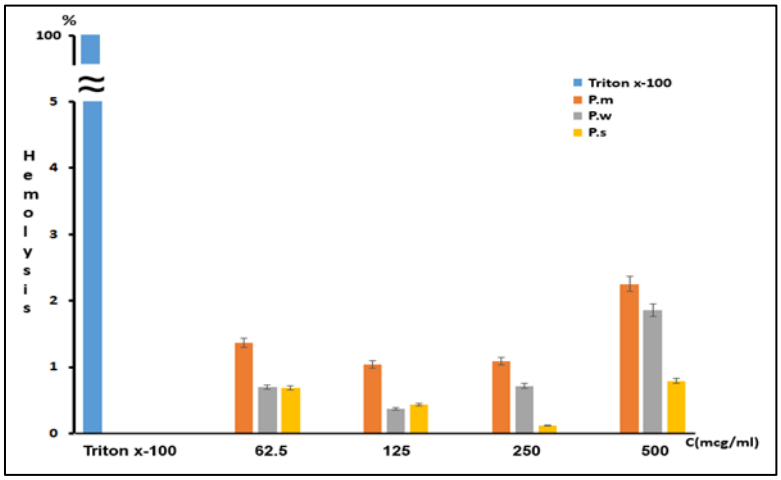

Figure 2 Hemolytic activity of P.m.3, P.w.3 and P.s.3

3.3. Isolation and Identification of Clitorin

The major compound was isolated from 50% methanolic fraction (P.w.3) using preparative HPLC. NMR analysis revealed the structure of the compound as clitorin, a glycosylated form of kaempferol, which exhibited a molecular formula of $C_{33}H_{40}O_{19}$ with a molecular weight of 740.66 g/mol (Fig. 3.)

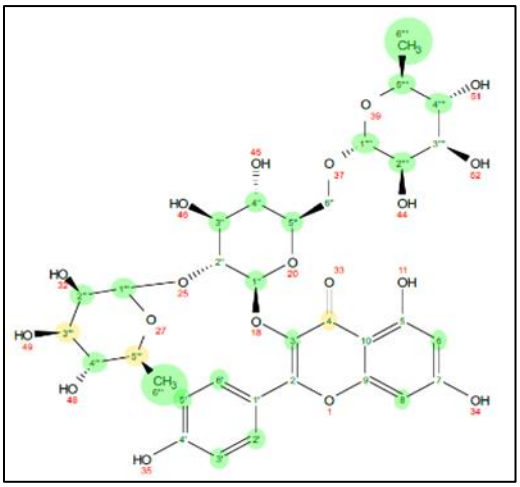

Figure 3 Chemical structure of clitorin (kaempferol 3-O-(2'',6''-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside)

^{1}H NMR spectrum identified an aromatic A_2B_2 spin system for the B-ring, with signals at δH 8.01 (2H, d, J = 8.8 Hz, H-2′,6′) and 6.89 (2H, d, J = 8.8 Hz, H-3′,5′), alongside two singlets at δH 6.35 (1H, s, H-8) and 6.17 (1H, d, J = 1.6 Hz, H-6), which corresponded to the protons of the A-ring. Proton signals of the sugar moieties appeared in the region of δH 3.27–5.60, including anomeric protons at δH 5.60 (1H, d, J = 7.5 Hz, H-1″), 5.25 (1H, s, H-1‴), and 4.52 (1H, m, H-1⁗), suggesting that it contained three sugar units. The two methyl doublets at δH 1.10 and 1.01 (each 3H, J = 6.2 Hz, d) indicated two terminal rhamnose units. This was also confirmed by the ^{13}C-NMR, where resonances at δC 99.1 (C-1″), 100.9 (C-1‴, C-1⁗), typical sugar carbons between δC 66.9–78.4 and methyl carbon at δC 16.2 and 16.5 are visible. These findings established the existence of one β-D-glucopyranosyl unit and two α-L-rhamnopyranosyl units. Similarly, the glucose at the C-3 position of kaempferol was confirmed by HMBC correlations between H-1″ and C-3. The chemical structure of the individual compound was as kaempferol 3-O-(2″,6″-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside, clitorin [35], established by TLC and NMR (^{1}H, ^{13}C, HSQC, HMBC) spectra and MS analysis. ^{1}H and ^{13}C NMR assignments for clitorin glycoside and its aglycone are presented in Table 4.

Table 4 ^{1}H and ^{13}C NMR Assignments for clitorin

Aglycon

C-atom	δC (ppm)	δH (ppm)
2	-	157.1
3	-	132.9
4	-	177.8
5	-	161.7
6	6.17 (d, 1.6 Hz, 1H)	98.7
7	-	165.0
8	6.35 (s, 1H)	93.5
9	-	157.5
10	-	104.4
1’	-	121.8
2’	8.01 (d, 8.8 Hz, 1H)	130.7
3’	6.89 (d, 8.8 Hz, 1H)	114.8
4’	-	159.8
5’	6.89 (d, 8.8 Hz, 1H)	-
6’	8.01 (d, 8.8 Hz, 1H)	-

Glycosides

- **Glycose**

C-atom	**δC (ppm)**	**δH (ppm)**
1''	5.60 (d, 7.5 Hz, 1H)	99.1
2''	3.57	77.6
3''	3.37	75.6
4''	3.26	72.4
5''	3.60	78.4

- **Rhamnose I**

C-atom	δC (ppm)	δH (ppm)
1'''	5.25	101.3
2'''	4.03 (m, 1H)	71.0
3'''	3.83	71.0
4'''	3.38	72.7
5'''	4.10	68.6
6'''	1.01	16.2

- **Rhamnose II**

C-atom	δC (ppm)	δH (ppm)
1''''	4.52 (m, 1H)	100.9
2''''	3.60	70.9
3''''	3.44	68.4
4''''	3.27	70.6
5''''	3.50	70.9
6''''	1.10	16.5

3.4. Quantification of Clitorin via HPLC

Quantitative analysis showed that clitorin was enriched in fraction P.w.3 (16.93 ± 1.16%, RSD), with an LOD of 0.00158897 and an LOQ of 0.00529656, compared to the crude extract P.w.1 (2.86 ± 2.5%, RSD; LOD 0.00073626; LOQ 0.00245421). Clitorin was not detected in fractions P.w.2 and P.w.4. These results confirm that the fractionation process effectively concentrates clitorin and support the validity of the preparative method and its resulting chemical profile.

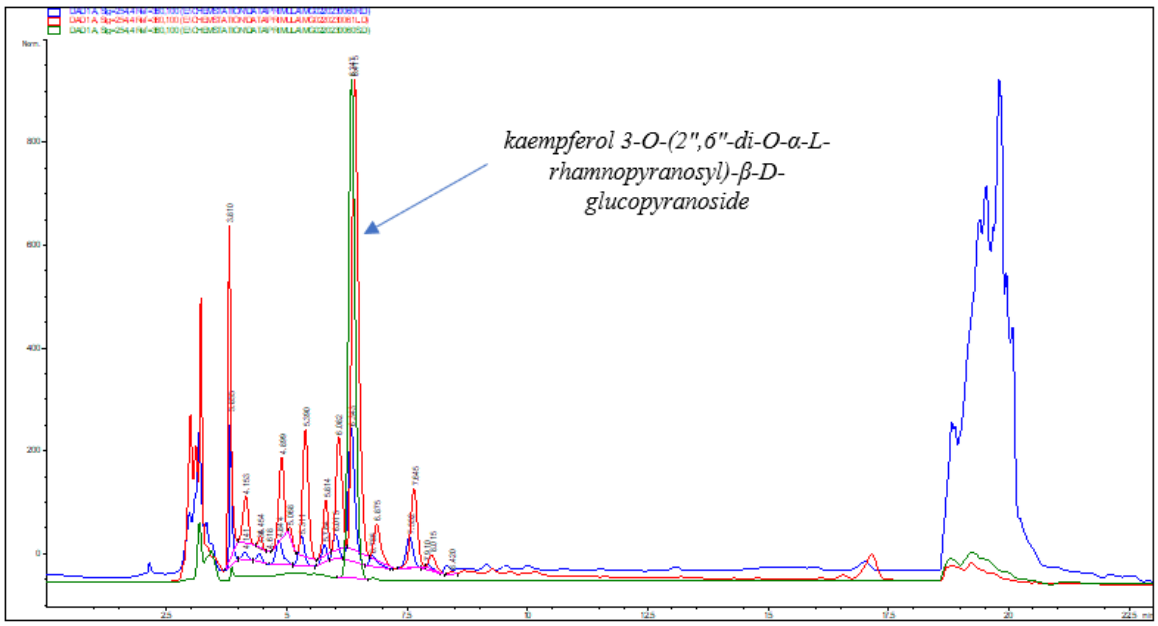

Figure 4 HPLC chromatogram of P.w 1 P.w 3 kaempferol *3-O-(2″,6″-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside*

3.5. In Silico Pharmacological Profile of Clitorin

The results of in silico assessement of clitorin using the PASS and GUSAR platforms are given in tables 5 and 7.

Table 5 PASS predicted biological activities of clitorin

Pa	Pi	Activity	Pa	Pi	Activity
0,957	0,002	Membrane permeability inhibitor*	0,848	0,007	Antineoplastic
0,926	0,005	Membrane integrity agonist*	0,839	0,005	CYP3A4 inducer
0,880	0,003	Anticarcinogenic	0,827	0,003	Chemopreventive
0,873	0,005	Anaphylatoxin receptor antagonist	0,822	0,003	Lipid peroxidase inhibitor*

0,871	0,007	HIF1A expression inhibitor	0,817	0,004	Antifungal
0,858	0,004	Vasoprotective*	0,801	0,003	Free radical scavenger*
0,853	0,010	CYP2H substrate	0,804	0,006	CYP3A inducer

* Activities, that coincide with the results obtained in in vivo experiment [24].

Table 6 PASS predicted protein targets* for the clitorin

Target name	Confidence	ChEMBL id
Ribosomal protein S6 kinase alpha 3	0.8519	CHEMBL2345
Serine/threonine-protein kinase NEK6	0.6449	CHEMBL4309
Protein kinase N1	0.4541	CHEMBL3384
Phosphatidylinositol-4-phosphate 5-kinase type-1 gamma	0.4261	CHEMBL1908383

* https://www.way2drug.com/passtargets/process.php

Table 7 Rat acute toxicity of clitorin (mg/kg) predicted by GUSAR

Route of administration	Median lethal dose (LD_{50}, mg/kg)	Toxicity according to OECD classification
Intraperitoneal	117.60	Class 4
Intravenous	283.70	Class 4
Oral	3151.00	Class 5
Subcutaneous	40.87	Class 3

Route of administration: IP – Intraperitoneal; IV – Intravenous; Or - Oral; SC – Subcutaneous.

PASS and GUSAR analyses predicted clitorin to possess a broad range of pharmacological activities, including antioxidant, antineoplastic, vasoprotective, chemopreventive, and anti-inflammatory properties. Notably, predicted targets include protein kinases such as ribosomal protein S6 kinase and NEK6, which play role in proliferation and stress response pathways. Additionally, clitorin is suggested to have low toxicity (Class 5), especially if administered orally, supporting its potential safety for further applications. Despite the aforementioned, further in vitro and in vivo assays are needed to prove in silico predictions.

3.6. Antioxidant capacity of Extracts and Clitorin

The antioxidant activity was evaluated using the ABTS assay. IC_{50} values demonstrate the following order of activity: gallic acid (G.A) 0.6624 ± 0.11; P.w.3 7.35 ± 0.23 µg/mL; Clitorin 13.25 ± 0.24 µg/mL; P.w.1 42.01 ± 0.21 µg/mL (Fig. 5).

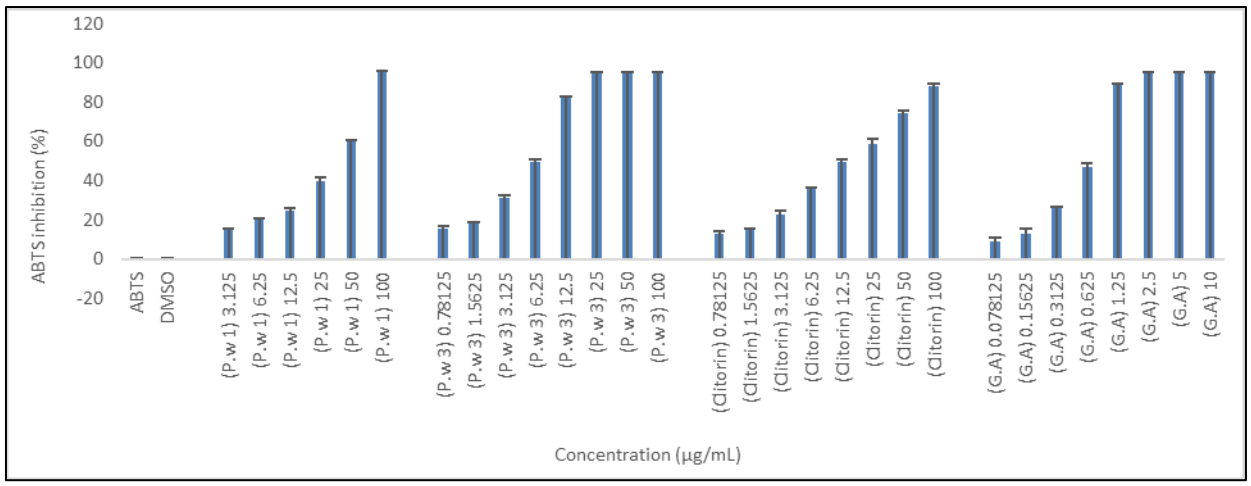

Figure 5 Inhibion of ABTS radical cation by P.w.1, P.w.3, Clitorin and gallic acid (G.A) (Mean ± SD)

3.7. Total Phenolic and Flavonoid Content in Extracts and Fractions

P.w.3 also possessed the highest total phenolic (41.84 ± 0.08 mg GAE/g) and flavonoid (36.02 ± 0.2 mg QE/g) content among all tested samples. This supports the ABTS assay findings and reinforces that polyphenolic compounds are directly responsible for antioxidant activity. The observed IC_{50} value of 7.35 ± 0.23 µg/mL indicates that P.w.3 exhibits very potent radical scavenging ability, which can be attributed to the high abundance of hydroxylated aromatic structures present in phenolics and flavonoids. These compounds can donate hydrogen atoms or electrons to neutralize ABTS radicals, while also potentially chelating redox-active metal ions, thereby preventing ROS formation.

Furthermore, the strong correlation between the total phenolic and flavonoid contents and the low IC_{50} suggests that the antioxidant activity is largely driven by these polyphenols. Flavonoid glycosides present in P.w.3 may also contribute to the stability and solubility of the active compounds, enhancing their radical scavenging efficiency. The combination of both high phenolic and flavonoid levels likely produces a synergistic effect, resulting in superior antioxidant activity compared to other fractions.

These findings are consistent with previous studies reporting that Primula species with enriched polyphenolic fractions demonstrate potent antioxidant activity in ABTS and other radical scavenging assays. Thus, P.w.3 can be considered the most promising fraction among the tested samples in terms of mitigating oxidative stress, which may have implications for its potential use in nutraceutical or pharmaceutical applications.

Table 8 Total Phenolic and Flavonoid Content (Mean ± SD)

N	Fraction	Phenolics (mg GAE/g)	Flavonoids(mg QE/g)
1	P.m.1	9.632813 ± 0.12	8.94 ± 0.2
2	P.w.1	12.82 ± 0.18	11.44 ± 0.15
3	P.s.1	10.67 ± 0.2	9.874 ± 0.18
4	P.m.3	38.38 ± 0.21	34.42 ± 0.21
5	P.w.3	41.84 ± 0.08	36.02 ± 0.2
6	P.s.3	37.68 ± 0.11	35.18 ± 0.09

These results confirm that P.w.3 is the most potent antioxidant compared to isolated clitorin. This suggests a synergistic effect between clitorin and other polyphenolic compounds present in the fraction. The correlation between total phenolic/flavonoid content and antioxidant activity again validates P.w.3 as a nontoxic, pharmacologically rich fraction.

4. Conclusion

It has been reported that family Primula L. species are rich in flavonoids and triterpene saponins, which have been proven to exhibit antioxidant and anti-inflammatory effects in *in vitro* experiments [23]. In the present investigation, further pharmacological studies of the crude extracts and their fractions supported these results and extended the knowledge of their biological actions. Among the tested fractions, P.m.4, P.w.4, and P.s.4 (100% methanol fractions) were identified as the most potent in eliminating both normal and cancerous cells, likely due to their significant triterpene saponin content. Cytotoxicity has been one of the commonly reported activities of triterpene saponins, which can be attributed to their membrane-disrupting properties due to their surfactant nature, apoptosis by caspase activation, and the mitochondrial pathway, as well as the inhibition of pro-inflammatory mediators like COX-2 and LOX. Additionally, both aglycone and glycone forms of saponins are crucial in all bioactivities [36]. However, since the 100% MeOH fractions also had cytotoxicity against normal cells, it is necessary to carry out further studies to evaluate systematic dose-response, long-term toxicity, and therapeutic safety margins before moving these fractions to clinical application.

On the other hand, the 50% methanol fraction of the extract of *P. woronowii* (P.w.3) was safer than the other fractions, with no cytotoxic or hemolytic activity, and exhibited superior antioxidant activity. Accordingly, this fraction was selected for the isolation of the compounds. Clitorin, a glycoside of kaempferol, was isolated and identified as the major compound (16.93% of the P.w.3 fraction). Clitorin showed good antioxidant activity by itself but revealed lower effects than fraction, suggesting the existence of a possible synergistic antioxidative effect with other phenolic compounds. This highlights the significance of compound–compound interactions within the fraction, which may enhance radical

scavenging through complementary mechanisms such as metal chelation, hydrogen donation, and stabilization of reactive intermediates. The total phenolic and flavonoid content, as well as the antioxidant potential, were closely related, indicating correlation and making P.w.3 a pharmacologically valuable plant. These results suggest that P.w.3 not only provides a high yield of active flavonoids but also represents a complex phytochemical matrix where additive or synergistic effects contribute to its overall pharmacological potential. Furthermore, in silico pharmacological profiling of clitorin using PASS and GUSAR predicted a broad spectrum of bioactivities, including antioxidant, antineoplastic, vasoprotective, chemopreventive, and anti-inflammatory effects. Predicted molecular targets, such as ribosomal protein S6 kinase and NEK6, suggest modulation of proliferation and stress-response pathways. GUSAR analysis also indicated low oral toxicity (Class 5), supporting the compound's potential safety for further preclinical development. Collectively, these results reinforce that clitorin is a promising multi-target bioactive flavonoid with both therapeutic potential and favorable safety characteristics, complementing the observed in vitro activities of the P.w.3 fraction. Taken together, the findings of the present investigation highlight *P. woronowii*, particularly the 50% methanolic fraction, as a potential source of promising therapeutically interesting bioactive flavonoids. An agent such as clitorin has definitely emerged as an essential candidate in these activities and a lead compound for further *in vivo* studies. This research not only provides support for the traditional medicinal uses of Primula species but also emphasizes their potential for inclusion in modern phytopharmaceutical formulations focusing on oxidative stress, inflammation, and associated conditions. Finally, integrating detailed phytochemical profiling with mechanistic, in vitro, in silico, and eventual in vivo studies strengthens the translational potential of *P. woronowii* and its constituents for development into safe and effective natural therapeutics.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no financial or any other conflicts of interest in this work.

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