

Topical anti-inflammatory activity of 2 α -hydroxy pentacyclic triterpene acids from the leaves of *Ugni molinae*

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Abstract—Leaf extracts of *Ugni molinae* Turcz. are used in the Chilean cosmetic industry on the assumption that they have decongestant, regenerative, and anti-aging properties. A bioassay-guided fractionation of this plant material showed that some extracts have potent anti-inflammatory activities. Further fractionation led to the isolation and identification of betulinic acid, a mixture of ursolic and oleanolic acids, and the 2 α -hydroxy derivatives alphitolic, asiatic, and corosolic acids. The latter three were evaluated in vivo in the mouse ear assay for their topical anti-inflammatory activity, inducing inflammation with either arachidonic acid (AA) or 12-*O*-tetradecanoylphorbol-13 acetate (TPA). Only corosolic acid was active in the AA assay, with similar potency to nimesulide, but all three triterpene acids inhibited TPA-induced inflammation with potencies comparable to that of indomethacin.

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1. Introduction

Ugni molinae Turcz. (*Myrtus ugni* Mol., Myrtaceae) is a shrub commonly known in the English-speaking countries as “Chilean guava”, in Spanish as murta or murtila, and as uñi in the Mapuche language of its native habitat. This plant, which grows wild in south-central Chile, is highly appreciated because of the pleasant flavor of its edible fruits, which are said to have been a favorite of Queen Victoria. Chilean folk medicine attributes to the aerial parts of this species astringent, stimulant, and aromatic properties,^{1,2} and it is believed to be helpful in the treatment of diseases of the urinary tract.³ Leaf extracts of the plant are used in cosmetics that are claimed to decongest, regenerate the skin, and

neutralize oxidative stress to retard the signs of aging. There is little information about the efficacy or the chemical composition of *U. molinae* leaves, but an analysis of the antioxidant polyphenols has been published quite recently.⁴ Extraction of the leaves in our laboratories with increasingly polar solvents, followed by anti-inflammatory assays, showed stronger activity in the hexane, dichloromethane, and ethyl acetate extracts than in that obtained subsequently with methanol. Further fractionation allowed us to identify the active constituents as triterpene acids.

2. Results and discussion

Ugni molinae leaves were extracted successively with hexane, dichloromethane, ethyl acetate, and methanol. As the crude hexane (H) and dichloromethane (DCM) extracts were highly colored, pigments were removed by preliminary clean-up using silica gel and Sephadex LH-20 columns in the former and only LH-20 in the

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latter case. Topical anti-inflammatory activity was assessed in the mouse ear model, inducing edema with either arachidonic acid (AA, 2 mg/ear) or 12-*O*-tetradecanoylphorbol acetate (TPA, 5 µg/ear).^{5,6} The purified H and DCM extracts and the crude ethyl acetate (EA) and methanol (M) extracts, at 1 mg/ear, showed strong anti-inflammatory activity versus TPA similar to the effect of indomethacin (0.5 mg/ear). At the same concentration, the H and EA extracts also reduced inflammation caused by AA to a similar extent as nimesulide (1 mg/ear), while the M extract was almost inactive and no significant activity could be demonstrated versus AA for the DCM extract.

The different extracts were subjected to TLC, developing with Liebermann–Burchard and anisaldehyde/sulfuric acid reagents, and observing them under long-wave UV light after exposure to ammonia vapors or natural products-polyethylene glycol reagent. The first two reagents showed that all extracts contained triterpenoids, steroids and/or saponins, and the latter two treatments indicated the presence of flavonoids in the EA and M extracts. Selective color reactions showed the presence of tannins in the methanol extract.

The purified H extract contained a mixture of low-polarity Liebermann–Burchard- and anisaldehyde/sulfuric acid-reactive compounds, and was not studied further due to its relatively small amount and complexity.

Fractionation of the DCM extract led to the isolation of betulinic acid, a mixture of oleanolic and ursolic acids, alphitolic (2 α -hydroxybetulinic), and corosolic (2 α -hydroxyursolic) acids (Fig. 1). Both alphitolic and corosolic acids showed similar activity against TPA-induced inflammation, but only corosolic acid, which was also purified from the M extract, inhibited AA-induced inflammation to the same degree as nimesulide, used as a reference compound in this test (Table 1). Fractionation of the EA extract concentrated on a compound that was as active as indomethacin versus TPA and inactive versus AA. This substance proved to be asiatic (2 α ,3 β ,23-trihydroxyurs-12-en-28-oic) acid (Fig. 1).

However, TLC of the DCM, EA, and M extracts suggested that all three contained the same triterpene acids, albeit in different proportions, with oleanolic/ursolic acids predominating in the DCM extract and increasing relative amounts of corosolic and asiatic acids in the EA and M extracts.

A number of triterpene acids with the ursane, oleanane, and lupane skeletons are known for their potent anti-inflammatory activity, which compares well with that of synthetic non-steroidal antiinflammatory drugs.^{7,8} Several recent reports on the topical anti-inflammatory activities of plant extracts have ascribed them to these compounds, and more specifically to the very widespread and relatively abundant ursolic acid.^{9–14}

Table 1. Topical anti-inflammatory effects induced by AA or TPA of extracts, isolated compounds of *Ugni molinae*, and reference drugs

Sample or reference drug	Dose (mg/ear)	% AE _{AA}	% AE _{TPA}
H extract	1	64.7 ± 7.7	88.4 ± 5.7
DCM extract	1	1.5 ± 10.1*	85.0 ± 2.9
EA extract	1	48.1 ± 4.7	83.1 ± 3.2
M extract	1	14.3 ± 8.2	78.3 ± 11.8
Alphitolic acid	0.5	2.4 ± 20.2*	92.4 ± 1.6
Corosolic acid	1	47.7 ± 6.9	n.d.
	0.5	n.d.	93.3 ± 2.1
Asiatic acid	1.6	n.d.	88.6 ± 2.7
	0.8	2.9 ± 12.9*	92.5 ± 2.0
	0.4	n.d.	77.6 ± 11.5
	0.2	n.d.	70.8 ± 10.7
	0.1	n.d.	67.2 ± 13.0
	0.05	n.d.	54.7 ± 11.1
	0.03	n.d.	34.7 ± 11.0
	0.025	n.d.	5.1 ± 17.2*
Nimesulide	1	48.8 ± 3.9	n.d.
Indomethacin	0.5	n.d.	92.9 ± 3.2

H, hexane; DCM, dichloromethane; EA, ethyl acetate; M, methanol. % AE_{AA} and AE_{TPA} correspond to topical anti-inflammatory effects induced by AA and TPA, respectively. Each group represents the median ± SEM of eight animals treated with samples or reference drugs. Without asterisks $p \leq 0.05$; * $p > 0.05$ (Wilcoxon); n.d., not determined.

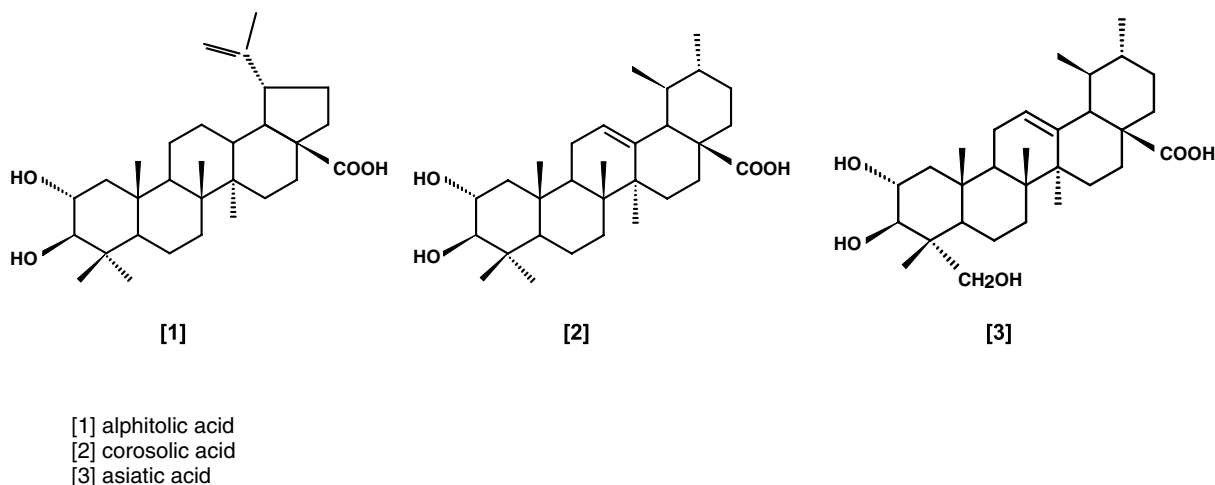


Figure 1. Alphitolic, corosolic, and asiatic acid structures.

Inhibition of phorbol ester-induced changes was demonstrated several years ago for corosolic acid¹⁵ (Ahn et al., 1998) and other pentacyclic triterpene acids (Huguet et al., 2000).¹⁶ More recently, corosolic acid, among many other triterpene acids, was shown to be a strong inhibitor of TPA-induced inflammation in the mouse ear assay.^{13,14} Moreover, the results reported in the two latter papers suggest that the introduction of additional hydroxyl groups on the oleanane and ursane scaffolds at C-2 and/or C-23 can be associated with increased anti-inflammatory activity. Such activities have not been reported previously for alphitolic and asiatic acids. Asiatic acid inhibited the TPA-induced edema in a dose-dependent manner ($EC_{50} = 0.1 \mu\text{mol/ear} = 0.05 \text{ mg/ear}$). The maximum inhibitory effect ($92.5 \pm 2.0\%$) was observed with $1.6 \mu\text{mol}$ of asiatic acid/ear (0.8 mg/ear), which did not differ significantly from that obtained with the same dose of indomethacin (Fig. 2).

Although we have not yet carried out detailed dose-response analyses, the fact that in our hands alphitolic acid, as well as corosolic acid and the reference compound indomethacin, inhibited TPA-induced inflammation by about 93% at doses of 0.5 mg/ear seems to confirm this trend, extending it to the lupane skeleton. A recent paper reported that $2\beta,3\alpha,23$ -trihydroxyurs-12-en-28-oic acid (one of the “esculentic” acids of the literature) and pomolic acid (19α -hydroxyursolic acid), at a concentration of $0.1 \mu\text{g/mL}$, inhibit IL-8 production elicited by $\text{TNF-}\alpha$ in cell culture to the same extent as sulfasalazine at this concentration, suggesting an additional mechanism of anti-inflammatory activity; however, the 2α -hydroxylated pentacyclic nortriterpene acids ilekudinol A and B, ursolic, corosolic, and asiatic acids were less active in this assay.¹⁷ As stated above, corosolic acid was also a potent inhibitor of AA-induced inflammation (Table 1).

Asiatic acid is well known for its wound-healing properties, related to its ability to stimulate collagen synthe-

sis,^{18,19} via changes in gene expression.²⁰ Also of dermatological interest are its antibacterial activity,²¹ and its ability to induce apoptosis in melanoma and other cancer cells.^{22,23} The anti-inflammatory effect of a semisynthetic asiatic acid derivative has been attributed to macrophage apoptosis,²⁴ but the fact that asiatic acid itself is a potent inhibitor of TPA-induced inflammation adds to its appeal as a constituent of dermatological preparations.

In summary, the strong topical anti-inflammatory activities of the purified H and DCM and the crude EA and M extracts of *U. molinae* leaves are ample justification for the use of this plant material as an ingredient in dermatological preparations. These results also provide a basis for the optimization of extraction methods and for the standardization of *U. molinae* leaf extracts. The anti-inflammatory activities are due mainly to the presence of several pentacyclic triterpene acids, including the 2α -hydroxy derivatives alphitolic, asiatic, and corosolic acids. The effects of alphitolic and asiatic acids on TPA-induced inflammation and of corosolic acid on inflammation elicited by AA are reported here for the first time.

3. Experimental

3.1. General procedures and chemicals

Precoated Merck silica gel 60F-254 plates were used for thin-layer chromatography (TLC) and the spots were detected using *p*-anisaldehyde–sulfuric acid mixture to detect triterpenoids.

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and arachidonic acid (AA) were purchased from Sigma Chem. (St. Louis, MO). Indomethacin and nimesulide were donated by Laboratorio Chile (Santiago, Chile). Analytical grade inorganic reagents and solvents were purchased from Merck (Darmstadt, Germany).

3.1.1. Plant material. *Ugni molinae* leaves were collected in April, 2002, in Chanco, south-central Chile, ($35^{\circ}45'S$, $72^{\circ}33'W$) and botanically identified by Dr. Carla Delporte. A voucher specimen was deposited in the herbarium of the School of Chemical and Pharmaceutical Sciences, University of Chile (SQF:22230).

3.1.2. Extraction, fractionation procedure, isolation, and identification of triterpene acids. Dried and ground leaves (200 g) were successively extracted by maceration at room temperature with hexane, CH_2Cl_2 , EtOAc, and MeOH; after removing the solvents in vacuo, the yields of the dry extracts, expressed as g/100 g dried leaves, were 1.75, 8.0, 5.8, and 20.4 for the hexane (H), dichloromethane (DCM), ethyl acetate (EA), and methanol (M) extracts, respectively.

The DCM extract (10 g) was fractionated over a silica gel column (column A), eluting with $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ mixtures of increasing polarity. Upon concentration, the fractions eluted with 97:3 and 96:4 (v/v) $\text{CH}_2\text{Cl}_2/$

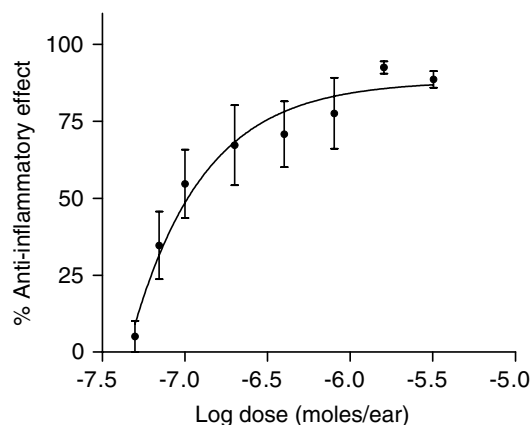


Figure 2. Topical anti-inflammatory effect of asiatic acid versus TPA-induced inflammation of the mouse ear. Data represents the median \pm SEM of the results for eight animals treated with asiatic acid. All values were significantly different from the control considered as 100% inflammation ($p < 0.05$, Wilcoxon).

EtOAc gave precipitates which were identified as betulinic acid (190 mg) and a mixture of oleanolic and ursolic acids (1.1 g), respectively. The fractions eluted with 90:10 and 80:20 to 60:40 CH₂Cl₂/EtOAc were concentrated separately and rechromatographed on silica gel (columns B and C, respectively), eluting with the same mixtures of solvents. Column B, eluted with 95:5 CH₂Cl₂/EtOAc, gave a precipitate that, purified and crystallized, was identified as alphitolic acid (450 mg). Column C, eluted with 90:10 CH₂Cl₂/EtOAc, gave a precipitate which was purified by additional column chromatography on silica gel, eluting with EtOAc, to afford corosolic acid (57.4 mg). The EA extract (3.0 g) was chromatographed on a polyamide column with water–MeOH mixtures of decreasing polarity. The fractions were monitored by TLC (CH₂Cl₂/EtOAc = 2:8), and the spots were detected by heating the plates at 110 °C after spraying with *p*-anisaldehyde–sulfuric acid. The fraction eluted with 25% water in MeOH (v/v) was rechromatographed on silica gel, eluting with CH₂Cl₂/EtOAc = 6:4, to give 1.2 g of a compound identified as asiatic acid.

The purity (>95%) and identity of the isolated compounds were confirmed by chromatography (TLC, HPLC) and by NMR (¹H, ¹³C, DEPT, and 1D-selective TOCSY experiments and two-dimensional HH-COSY, HSQC, and HMBC experiments, as necessary).

3.1.3. Betulinic acid. ¹H NMR (CDCl₃–DMSO-*d*₆) δ (ppm) 0.83 (1H, m, H-1α), 1.57 (1H, m, H-1β), 1.45 (2H, m, H-2), 2.98 (1H, dd, *J* = 5.0, 11.2 Hz, H-3), 0.60 (1H, m, H-5), 1.44 (1H, m, H-6α), 1.32 (1H, m, H-6β), 1.31 (2H, m, H-7), 1.22 (1H, m, H-9), 1.36 (1H, m, H-11α), 1.18 (1H, m, H-11β), 0.96 (1H, m, H-12α), 1.62 (1H, m, H-12β), 2.24 (1H, m, H-13), 1.08 (2H, m, H-15), 1.33 (1H, m, H-16α), 2.14 (1H, m, H-16β), 1.49 (1H, m, H-18), 2.96 (1H, ddd, *J* = 4.5, 10.6 Hz, H-19), 1.32 (1H, m, H-21α), 1.82 (1H, m, H-21β), 1.38 (1H, m, H-22α), 1.82 (1H, m, H-22β), 0.87 (3H, s, H-23), 0.65 (3H, s, H-24), 0.76 (3H, s, H-25), 0.87 (3H, s, H-26), 0.92 (3H, s, H-27), 4.66 (1H, d, *J* = 1.8 Hz, H-29α), 4.53 (1H, d, *J* = 1.8 Hz, H-29β), 1.63 (3H, s, H-30).

¹³C NMR (CDCl₃–DMSO-*d*₆) δ (ppm) 38.82 (C-1), 27.58 (C-2), 77.40 (C-3), 38.93 (C-4), 55.42 (C-5), 18.41 (C-6), 34.42 (C-7), 40.74 (C-8), 50.48 (C-9), 37.19 (C-10), 20.94 (C-11), 25.53 (C-12), 38.06 (C-13), 42.43 (C-14), 29.67 (C-15), 32.26 (C-16), 55.88 (C-17), 49.11 (C-18), 47.00 (C-19), 150.60 (C-20), 30.62 (C-21), 37.00 (C-22), 28.46 (C-23), 16.16 (C-24), 16.34 (C-25), 16.09 (C-26), 14.82 (C-27), 177.68 (C-28), 109.86 (C-29), 19.42 (C-30).

3.1.4. Oleanolic and ursolic acids. Spectral data were in agreement with published data.²⁵

3.1.5. Alphitolic acid. ¹H NMR (DMSO-*d*₆) δ (ppm) 3.41 (1H, ddd, *J* = 4.4, 9.3, 11.3 Hz, H-2), 2.72 (1H, d, *J* = 9.3 Hz, H-3), 2.22 (1H, ddd, *J* = 3.4, 12.7, 12.7 Hz, H-13), 1.52 (1H, t, *J* = 11.3 Hz, H-18), 2.96 (1H, m, H-19), 0.90 (3H, s, H-23), 0.68 (3H, s, H-24), 0.82 (3H, s, H-25), 0.87 (3H, s, H-26), 0.94 (3H, s, H-27),

4.70 (1H, d, *J* = 1.8 Hz, H-29α), 4.57 (1H, d, *J* = 1.8 Hz, H-29β), 1.66 (3H, s, H-30).

¹³C NMR (DMSO-*d*₆) δ (ppm) 47.57 (C-1), 67.82 (C-2), 82.64 (C-3), 39.41 (C-4), 55.36 (C-5), 18.46 (C-6), 34.32 (C-7), 40.77 (C-8), 50.36 (C-9), 38.31 (C-10), 21.07 (C-11), 25.49 (C-12), 38.06 (C-13), 42.52 (C-14), 30.61 (C-15), 32.18 (C-16), 55.89 (C-17), 49.02 (C-18), 47.10 (C-19), 150.77 (C-20), 29.63 (C-21), 36.79 (C-22), 29.12 (C-23), 17.35 (C-24), 17.59 (C-25), 16.22 (C-26), 14.84 (C-27), 177.66 (C-28), 110.07 (C-29), 19.44 (C-30).

3.1.6. Corosolic acid. ¹H NMR (CD₃OD) δ (ppm) 3.64 (1H, ddd, *J* = 4.7, 9.8, 11.3 Hz, H-2), 2.93 (1H, d, *J* = 9.5 Hz, H-3), 5.25 (1H, t, *J* = 3.4 Hz, H-12), 2.23 (1H, d, *J* = 11.3 Hz, H-18), 1.04 (3H, s, H-23), 0.83 (3H, s, H-24), 1.04 (3H, s, H-25), 0.87 (3H, s, H-26), 1.14 (3H, s, H-27), 0.91 (3H, d, *J* = 6.5 Hz, H-29), 0.99 (3H, s, H-30).

¹³C NMR (CD₃OD) δ (ppm) 46.87 (C-1), 68.10 (C-2), 83.06 (C-3), 39.09 (C-4), 55.26 (C-5), 18.12 (C-6), 32.81 (C-7), 39.41 (C-8), 47.55 (C-9), 37.78 (C-10), 23.04 (C-11), 125.31 (C-12), 138.35 (C-13), 41.89 (C-14), 27.76 (C-15), 23.89 (C-16), 47.55 (C-17), 52.95 (C-18), 39.00 (C-19), 39.00 (C-20), 30.36 (C-21), 36.68 (C-22), 27.91 (C-23), 16.09 (C-24), 15.80 (C-25), 16.39 (C-26), 22.69 (C-27), 180.20 (C-28), 16.22 (C-29), 20.15 (C-30).

3.1.7. Asiatic acid. ¹H NMR (CD₃OD) δ (ppm) 3.71 (1H, ddd, *J* = 4.4, 10.0, 4.7 Hz, H-2), 3.37 (1H, d, *J* = 9.6 Hz, H-3), 5.26 (1H, t, *J* = 3.4 Hz, H-12), 2.23 (1H, d, *J* = 11.3 Hz, H-18), 3.29 (1H, d, *J* = 11.1 Hz, H-23), 3.52 (1H, d, *J* = 11.1 Hz, H-23), 0.72 (3H, s, H-24), 1.07 (3H, s, H-25), 0.87 (3H, s, H-26), 1.15 (3H, s, H-27), 0.91 (3H, d, *J* = 6.4 Hz, H-29), 0.99 (3H, s, H-30).

¹³C NMR (CD₃OD) δ (ppm) 46.64 (C-1), 68.30 (C-2), 76.88 (C-3), 42.72 (C-4), 46.84 (C-5), 17.69 (C-6), 32.26 (C-7), 39.41 (C-8), 47.50 (C-9), 37.60 (C-10), 23.40 (C-11), 125.27 (C-12), 138.42 (C-13), 42.00 (C-14), 27.77 (C-15), 23.92 (C-16), 48.45 (C-17), 52.97 (C-18), 39.00 (C-19), 39.00 (C-20), 30.37 (C-21), 36.70 (C-22), 65.04 (C-23), 12.51 (C-24), 16.25 (C-25), 16.46 (C-26), 22.76 (C-27), 180.21 (C-28), 16.28 (C-29), 20.16 (C-30).

3.1.8. Animals. Adult male CF-1 mice (20–25 g), derived from a stock maintained at the Chilean Public Health Institute, were used to assess the anti-inflammatory effects. All animals were kept on a 14:10 h light–dark cycle, with water and food provided ad libitum and were fasted overnight before the experiments. All procedures were performed using protocols approved by the Ethics Committees of the Public Health Institute and of the Faculty of Chemical and Pharmaceutical Sciences.

3.1.9. Topical anti-inflammatory activity. The topical anti-inflammatory activity was evaluated in vivo as described by Delporte et al.⁶ Briefly, groups of 8 animals were treated with a single dose of extract (1 or 3 mg/

20 $\mu\text{L}/\text{ear}$), chromatographic fraction (1 mg/ear) or pure triterpene acid (0.025–1.56 mg/ear) dissolved in acetone and applied topically on the inner (10 μL) and outer (10 μL) surface of the right ear of the animals of each group. After 5 min, 2 mg of arachidonic acid (AA) or 5 μg 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was administered topically on the right ear and acetone on the left ear as a solvent control. Groups of control animals were treated similarly, but they did not receive the samples. Another two groups of 8 animals were treated with nimesulide or indomethacin, drugs used as references for topical inhibition of inflammatory activity induced by AA or TPA, respectively. After 1 and 4.5 h for AA and TPA, respectively; all the animals were sacrificed by cervical dislocation and a section of 6 mm diameter of the right and left ears was punched out and weighed. Data were expressed as median \pm SEM values calculated from the weight of the ear disks for treated and untreated animals considering control values as 100% inflammation. Drug-induced changes were evaluated using the Wilcoxon test for independent data and were considered statistically significant with $p \leq 0.05$.

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