

1 Article

2 The wide spectrum anti-inflammatory activity of 3 andrographolide in comparison to NSAIDs: a 4 promising therapeutic compound against the 5 cytokine storm

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15 **Abstract:** The challenges of the COVID-19 pandemic have highlighted an increasing clinical demand for safe
16 and effective treatment options against an overzealous immune defence response, also known as the “cytokine
17 storm”. Andrographolide is a naturally derived bioactive compound with promising anti-inflammatory activity
18 in many clinical studies. However, its cytokine-inhibiting activity, in direct comparison to commonly used
19 nonsteroidal anti-inflammatory drugs (NSAIDs), has not been extensively investigated in existing literature. The
20 anti-inflammatory activities of andrographolide and common NSAIDs, such as diclofenac, aspirin, paracetamol
21 and ibuprofen were measured on lipopolysaccharide (LPS) and interferon- γ induced RAW264.7 cells. The levels
22 of PGE₂, nitric oxide (NO), TNF- α & LPS-induced release of pro-inflammatory cytokines on differentiated
23 human macrophage THP-1 cells were measured against increasing concentrations of andrographolide and
24 aforementioned NSAIDs. The associated mechanistic pathway was examined on NF κ B using flow cytometry on
25 the human endothelial-leukocyte adhesion molecule (ELAM9) (E-selectin) transfected RAW264.7 cells with
26 green fluorescent protein (GFP). Andrographolide exhibited broad and potent anti-inflammatory and cytokine-
27 inhibiting activity in both cell lines by inhibiting the release of IL-6, TNF- α and IFN- γ , which are known to play
28 a key role in the etiology of cytokine storm and the pathogenesis of inflammation. In comparison, the tested
29 NSAIDs demonstrated weak or no activity against proinflammatory mediators except for PGE₂, where the
30 activity of andrographolide (IC₅₀ = 8.8 μ M, 95% CI= 7.4 to 10.4 μ M) was comparable to that of paracetamol (IC₅₀
31 = 7.73 μ M, 95% CI = 6.14 to 9.73 μ M). The anti-inflammatory action of andrographolide was associated with its
32 potent downregulation of NF κ B. The wide-spectrum anti-inflammatory activity of andrographolide
33 demonstrates its therapeutic potential against cytokine storms as an alternative to NSAIDs.

34 **Keywords:** Andrographolide; Anti-inflammatory; Cytokine storm; NSAID drugs; NF κ B

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42 1. Introduction

43 In light of the recent coronavirus disease SARS-CoV-2 (COVID-19) pandemic, the development
44 of immunomodulatory drugs has gained considerable interest from the public and the scientific
45 community. This interest has emerged due to the identification of "hyperinflammatory" acute
46 respiratory distress syndrome (ARDS) as the key driver of the severity and mortality of COVID-19,
47 which is supported by findings from early and recent clinical trials. [1-4]. In a range of auto-immune
48 and infectious conditions, immune-signalling proteins known as cytokines are released from the host
49 immune system, which can go into overdrive and trigger the uncontrolled surging levels of cytokine
50 release, the "cytokine storm" or cytokine release syndrome (CRS) [5]. The characteristic "cytokine
51 storm" of COVID-19 is also a primary feature in patients who experience sudden acute respiratory
52 syndrome (SARS) and middle-east respiratory syndrome (MERS), which are caused by other
53 coronaviruses [6]. Clinically, it commonly presents as systemic inflammation, multiple organ failure,
54 and high inflammatory parameters that can lead to patient mortality [7]. The recent (2023) uptick in
55 cases of respiratory syncytial virus (RSV) in Australia [8,9], especially among children, also has
56 cytokine storm associated with it [10] and is accompanied by even more dangerous secondary
57 complications such as encephalitis [11]. Hence, it is imperative to contemplate the utilisation of anti-
58 inflammatory therapies and immunosuppressive drugs that can target a broad range of inflammatory
59 mediators to prevent a fatal cytokine storm and hence adverse patient outcome. However, more
60 rigorous comparative studies, *in vitro* followed by *in vivo*, are needed to confirm the activity and
61 efficacy of any novel and off-label drugs [12].

62 Nonsteroidal anti-inflammatory drugs (NSAIDs) are the regular prescription for acute and
63 chronic inflammation-induced pain [13,14]. As a result, they are also being explored for managing
64 fever and hyperinflammation that can occur during viral infections. NSAIDs work by inhibiting
65 cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), thereby blocking the production of
66 prostaglandins, which are essential mediators of fever and inflammation. Ibuprofen, an NSAID
67 frequently prescribed, has demonstrated the ability to decrease interleukin-6 (IL-6) levels in human
68 tissues and sputum [15]. This observation aligns with outcomes from clinical trials conducted amid
69 the COVID-19 pandemic. These trials and more recent meta-analyses suggest that utilising ibuprofen
70 and other NSAIDs for anti-IL-6 therapies could be a viable option, since NSAIDs do not cause
71 increased rates of SARS-COV-2 infection or symptom severity when used for analgesic and
72 antipyretic treatment during COVID-19 [16,17]. However, caution is advised when using ibuprofen
73 and other NSAIDs to treat severe COVID-19 that requires hospitalisation [14,18]. Serious adverse
74 effects on the gastrointestinal (GI) and cardiovascular systems may limit the use of NSAIDs against
75 cytokine storm. For instance, diclofenac showed an increased risk of myocardial infarction, similar to
76 rofecoxib with compatible high COX-2 inhibitory potency [19], suggesting a link. NSAIDs may also
77 increase angiotensin-converting enzyme 2 expression, increase the viral load, and may worsen the
78 clinical outcomes [20]. Thus, due to the associated risks and adverse reactions, the use of NSAIDs
79 such as ibuprofen and acetaminophen for cytokine storm remains controversial [21].

80 Natural products and their derivatives have long formed "the backbone of modern
81 pharmacopoeias" [22,23]. There is an increasing realisation that synthetic approaches to drug
82 development have not lived up to their promise, thus renewing interest in natural products as drug
83 discovery sources [24]. Andrographolide is an ent-Labdane diterpenoid, and the primary bioactive
84 compound from *Andrographis paniculata*, a medicinal herb, has been used to treat a wide variety of
85 ailments linked to inflammation [25,26,27]. Andrographolide has been shown to inhibit a wide range
86 of inflammatory mediators and can be a therapeutic candidate for a wide range of inflammatory and
87 bacterial conditions, including rheumatoid arthritis, acute colitis, cigarette smoke-induced oxidative
88 lung injury, *Chlamydia trachomatis* infections, and in some instances of bacterial pneumonia [28-31].
89 Notably, andrographolide inhibited influenza A virus-induced inflammation in the C57BL/6 mice
90 model by reducing key cytokines of the cytokine storm, including IL-6, IL-10, TNF- α and interferon
91 (IFN)- γ via the downregulation on NF κ B and JAK-STAT signaling pathway [32].

92 The safety profile of andrographolide has been well established in literature and is considered
93 to quite safe [33]. Adverse events associated with andrographolide, and andrographolide-derivative
94 medications are extremely rare, but include gastrointestinal problems, skin and subcutaneous

disorders, and anaphylaxis. These adverse events are primarily associated with injections, oral consumption of andrographolide and andrographolide herbal extracts are essentially safe [34]. Therefore, andrographolide is a potential lead compound for the development of new anti-inflammatory compounds guided by historical use and not by specific COX inhibition. Interestingly, andrographolide has been reported to exhibit gastro-protective, and ulcer-preventive effects, which, combined with its well-documented anti-inflammatory effects, could make it a safe alternative to traditional NSAIDs [35].

This study aimed to investigate the effect of andrographolide on lipopolysaccharide (LPS) and IFN- γ induced inflammatory mediators and cytokines on macrophage and monocyte cells. The inhibitory effects of andrographolide in suppressing a range of cytokines were compared with popular NSAIDs such as aspirin, ibuprofen, diclofenac, and acetaminophen.

2. Materials and Methods

2.1. Chemicals and reagents

Andrographolide (C₂₀H₃₀O₅, purity >98%) was purchased from Biopurify Phytochemicals Ltd. (Chengdu, China), with reported purity certified by HPLC analysis. Ibuprofen sodium salt (98%), diclofenac (99%), acetaminophen (paracetamol, 99%), prednisone (98%) and dexamethasone (97%) analytical standards were purchased from Sigma-Aldrich (NSW, Australia).

Lipopolysaccharides (LPS) isolated from *Escherichia coli* strain 0111:B4, phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, 3,3,5,5-tetramethylbenzidine (TMB), dimethylsulfoxide (DMSO), and citric acid analytical standards were purchased from Sigma-Aldrich (NSW, Australia). The murine IFN- γ and murine TNF- α , and prostaglandin E2 (PGE2) enzyme-linked immunosorbent assay (ELISA) kit were purchased from Peprotech (NSW, Australia). Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) used to culture cells were obtained from Lonza (NSW, Australia). GlutaMax, penicillin, and streptomycin were purchased from Life Technologies (NSW, Australia). The foetal bovine serum (FBS) (French origin) was purchased from Bovogen Biologicals (VIC, Australia). The strep avidin horse radish peroxidase used in the TNF- α ELISA was purchased from BD Biosciences (NSW, Australia). The Bio-Plex Pro cytokine, chemokine and growth factor assay kits, human cytokine 17 and 27-plex, were purchased from Bio-Rad (NSW, Australia).

2.2. Cell culture

The murine macrophage RAW264.7 cells (from American Type Culture Collection (ATCC), VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) from Lonza (NSW, Australia), containing 4.5 g/L D-glucose and supplemented with 2 mM l-GlutaMax, 100 units/mL penicillin, 100 μ g/mL streptomycin (Life Technologies, Australia), and 5% FBS. The immortalized RAW264.7 monocyte or macrophage-like cells originate from the Abelson leukemia virus transformed cell line derived from BALB/c mice [36]. The immortalized RAW264.7 cell line was preferred over human/animal derived primary cell lines due to a cheaper-costs, ease of availability, and minimal ethical concerns [37]. The cells were incubated in a humidified atmosphere containing 5% CO₂ and 95% air. The human monocyte THP-1 cells were cultured in RPMI 1640 media from Lonza (NSW, Australia), containing 4.5 g/L D-glucose and supplemented with 2 mM GlutaMax, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% FBS from Life Technologies (NSW, Australia), at 37°C, 5% CO₂ in 95% air. Using PMA (100 nM) for 24 h, THP-1 cells were differentiated towards macrophage-like phenotype and subsequently seeded for the bioassays.

2.3. Protocol for MTT viability determination

A 100 μ L of MTT solution (0.2 mg/mL MTT in complete medium) was added to the cell culture and incubated for 2 h at 37 °C (5% CO₂). The MTT solution was removed, and 150 μ L of DMSO was added to dissolve the formazan crystals. It should be noted DMSO was used as the vehicle control, and no noticeable effect in the final analysis. It should also be noted that dexamethasone (97%) was used as the cytokine positive control. The plate was shaken for 5 min before absorbance was measured at 595 nm on a FLUOstar Omega microplate reader from BMG Labtech (VIC, Australia).

145 *2.4. Determination of nitric oxide release in LPS and IFN- γ stimulated RAW264.7 cells.*

146 Nitric oxide (NO) release was quantified using Griess reagent [38]. Briefly, the RAW264.7 cells
147 were seeded at 1×10^5 cells/well on a 96-well culture plate (Corning® Costar®, Sigma-Aldrich,
148 Australia) for 48 h. Andrographolide and the NSAIDs were dissolved in DMSO (final concentration
149 of 0.1% *w/v*), 1 h before stimulation with LPS and IFN- γ (50 ng/mL, 50 units/mL). After the cells were
150 co-incubated for 18 h, the supernatant was collected (180 μ L) and reacted with the Griess reagent (100
151 μ L) to quantify dissolved nitrates at 540 nm (colorimetry) on the FLUOstar microplate reader. The
152 remaining cells were tested with MTT solution to assess the cell viability.

153 *2.5. Determination of prostaglandin E2 and TNF- α in LPS and IFN- γ stimulated RAW264.7 cells*

154 Prostaglandin E2 (PGE2) release and TNF- α were quantified by commercial ELISA kits in
155 accordance with supplying manufacturers' protocol [39]. Briefly, the RAW264.7 cells were seeded at
156 1×10^5 cells/well in a 96-well plate for 48 h. The compounds of interest were dissolved in DMSO (final
157 concentration 0.1% *w/v*) 1 h before the stimulation with LPS and IFN- γ (50 ng/mL, 50 units/mL). After
158 18 h, the supernatant (180 μ L) was collected and subjected to ELISA assay.

159 *2.6. Determination of multiple cytokines in LPS-stimulated THP-1 cells using a Bioplex cytokine assay.*

160 The inhibitory effect on cytokines was tested in a panel of 17 inflammatory mediators using Bio-
161 Plex Pro cytokine, chemokine and growth factors assay kits, human cytokine 17 and 27-plex from
162 Bio-Rad, (NSW, Australia) on PMA-differentiated THP-1 cells. The 17 inflammatory mediators
163 include proinflammatory cytokines such as TNF- α , IFN- γ , IL-1 β , IL-5, IL-6, IL-7, IL-8, IL-12, G-CSF,
164 GM-CSF, MCP-1 and MIP-1b, and anti-inflammatory cytokines such as IL-2, IL-4, IL-9, IL-10 and IL-
165 13. Briefly, the cells were seeded at 1×10^5 cells/well in a 96-well plate for 48 h. The compounds of
166 interest were dissolved in DMSO (final concentration 0.1% *w/v*) for 1 h before the stimulation with
167 LPS (1 μ g/mL) for 6 h before the supernatant was harvested and stored (-80 °C) for analysis using the
168 bead-based assay. The experiments were conducted with the Bio-Plex 100 system from Bio-Rad
169 (NSW, Australia). The plates were washed using a 96-well plate magnetic handheld washer from Bio-
170 Rad (NSW, Australia). The Bio-Plex Manager 3.0 software was used to operate the system and
171 interpret the data.

172 *2.7. The regulation of NF- κ B signalling pathway using a FACS Canto II flow cytometer*

173 The RAW264.7 cells were stably transfected with the human endothelial-leukocyte adhesion
174 molecule (ELAM9) (E-selectin) promoter (-760 to +60 mV), driving destabilised enhanced green
175 fluorescent protein (GFP) [40,41]. The ELAM9 RAW264.7 cells used in the NF- κ B activation assay
176 were analysed using a BD FACS Canto II flow cytometer from Becton, Dickinson and Company
177 (NSW, Australia), equipped with a high throughput fluorescence-activated cell sorting (FACS) flow
178 autosampler with three laser sets (405, 488, 635 nm) and corresponding filter sets. After the co-
179 incubation with compounds of interest and LPS (50 μ g/mL) and IFN- γ (50 units/mL) for 5.5 h after
180 stimulation, the RAW264.7 cells were then washed with ice-cold (0 °C) PBS and harvested by trypsin.
181 After resuspension in PBS with 10% FBS, the cells were then filtered through a 50 μ m Nylon filter
182 into a new 96-well plate. The blue layer was used for the analysis of GFP in the ELAM9 RAW264.7
183 cells. The forward scatter (FSC) and side scatter (SSC) were determined based on the size and shape
184 of the control cells plated in each experiment. Readout of the laser intensity was normalised in each
185 experiment to the fluorescence of the normal RAW264.7 cells. The data was analysed using FlowJo
186 v10. The response was then normalised to the unstimulated and stimulated controls and expressed
187 as a percentage of stimulated untreated NF- κ B activation. The dose-response curves were constructed
188 in GraphPad Prism v5 (CA, USA) by plotting the log of the dose concentration against the percentage
189 release. A non-linear 4-parameter variable slope dose-response curve was fitted to calculate the IC₅₀
190 value for each sample tested.

191 *2.8. Statistical analysis*

192 All data is reported with the standard error of the mean (SEM) displayed as error bars in the
193 figures. The *in vitro* experiments were performed in triplicate, and the entire experiment was repeated
194 on three or more separate days ($n = 3$). Multicytokine assays were performed only once and in

195 duplicate due to their high cost, and the assay duplication ($n = 2$) is indicated in the dose-response
196 curves. The dose-response data was fitted with a log (inhibitor) *vs.* normalised response with a
197 variable slope model using GraphPad Prism v5 (CA, USA). The IC_{50} values were calculated from the
198 fitted curves. All fitted curves were constrained to a minimum of 0 and a maximum of 100. The
199 uncertain measurement in the IC_{50} values is expressed at the 95% confidence interval (CI) ($p = 0.05$).

200 3. Results and Discussion

201 3.1. Broad inhibitory effects of andrographolide on NO, PGE₂ and TNF- α in LPS and IFN- γ stimulated 202 RAW264.7 cells.

203 Andrographolide and common NSAIDs, including diclofenac, aspirin, paracetamol, ibuprofen
204 were tested on LPS and IFN- γ induced PGE₂, NO and TNF- α assays on RAW264.7 cells at multiple
205 concentration levels. The IC_{50} values for all tests are summarised in **Table 1**.

206 The results from **Figure 1** indicate that andrographolide exhibited a dose-dependent inhibitory
207 effect against PGE₂ ($IC_{50} = 8.8 \mu\text{M}$, 95% CI = 7.4 to 10.4 μM), with activity comparable to paracetamol
208 ($IC_{50} = 7.73 \mu\text{M}$, 95% CI = 6.14 to 9.73 μM). All tested NSAIDs demonstrated greater potency than
209 andrographolide, except for aspirin, with an IC_{50} value of 14.10 μM (**Figure 1A**). Therefore,
210 andrographolide inhibited PGE₂ production at about the same potency as weak non-selective
211 NSAIDs such as aspirin and paracetamol *in vitro*. Diclofenac and ibuprofen demonstrated more
212 potent PGE₂ inhibition, but they are not considered selective COX-2 inhibitors and long-term use is
213 associated with adverse gastrointestinal (GI) effects [26], whereas paracetamol and aspirin are
214 generally considered safer in the clinical context [42]. NSAIDs relieve pain and fever by inhibiting
215 the synthesis of PGE₂ *via* COX enzymes.

216 The inhibition of COX-2 leading to decreased proinflammatory cytokine levels and leukocyte
217 activation is considered therapeutically beneficial, whereas the inhibition of COX-1 is associated with
218 unwanted side effects partly attributed to the non-specific suppression of prostaglandins [35]. Since
219 COX-1 has been associated with improved survival in viral-infected hyperinflammatory conditions,
220 NSAIDs with non-specific COX inhibition are not recommended for therapeutic use in cytokine
221 release syndrome (CRS) as the first line of clinical treatment [43]. In contrast, andrographolide has
222 been shown to specifically inhibit COX-2 expression in human fibroblast cells under the stimulation
223 of LPS [44], and andrographolide sodium bisulfate is known to exert a gastroprotective effect against
224 indomethacin-induced gastric ulcer in rats *via* an increase of mRNA expression of COX-1 [45]. Thus,
225 the *in vitro* anti-PGE₂ activity of andrographolide is comparable to paracetamol as a potentially weak
226 pain killer, but it may present with reduced side-effects.

227 Type 2 nitric oxide synthase (iNOS or NOS2) is highly expressed in activated macrophages,
228 which plays a crucial role in the pathogenesis of inflammation [46]. Induced by IL-6 and IL-1, aberrant
229 NO production is directly involved in CRS pathogenesis and is known to cause vasodilation and
230 hypotension, which are standard features of clinical CRS that require vasopressor administration
231 [47]. In addition, both iNOS and PGE₂ have been found to contribute to pain, swelling and cartilage
232 destruction in inflammatory diseases such as those associated with the osteoarthritic joint [48]. As
233 shown in **Figure 1B**, andrographolide inhibited NO in a dose-dependent manner and exhibited
234 greater potency compared to the NSAIDs, with an IC_{50} value of 7.4 μM (95% CI from 6.7 to 8.1 μM).
235 The NSAIDs showed no significant NO inhibition until the concentration was increased to >100 μM .
236 Diclofenac displayed the highest potency with an IC_{50} value of 222 μM , which was still much higher
237 than that of andrographolide. This result is in line with a previous study that demonstrated that
238 andrographolide suppressed the expression of iNOS in macrophages and subsequently restored
239 vasoconstriction in rat aortas treated with LPS [49]. To date, no NOS inhibitors are available for the
240 treatment of inflammatory-induced pain. Andrographolide may serve as a potential therapeutic
241 compound in the role of a NOS blocker, which could be beneficial for cardiac vasodilation.

242 TNF- α is an important proinflammatory cytokine that plays a central role in the cytokine storm.
243 As seen in **Figure 1C**, andrographolide exhibited the most significant inhibition of TNF- α ($IC_{50} = 23.3$
244 μM) compared to NSAIDs, which did not show any significant TNF- α inhibition at the tested
245 concentrations. It should be noted that ibuprofen showed a weak TNF- α inhibition with an IC_{50}
246 estimated above 1500 μM .

247 As NSAIDs are not targeted at iNOS or TNF- α , it is not surprising that they show little to no
248 activity against these two therapeutic targets. On the other hand, andrographolide displayed potent

249 inhibition of both iNOS and TNF- α in addition to PGE₂, highlighting its broad anti-inflammatory
250 activity *via* different mechanisms [50]. In addition, the potent inhibitory activity on TNF- α of
251 andrographolide indicates its potential use against CRS. Therefore, further investigations were
252 undertaken on the cytokine-inhibiting activity of andrographolide on THP-1 cells.

253 **Table 1.** The half maximal inhibitory concentrations of andrographolide, diclofenac, aspirin, paracetamol, and
254 ibuprofen in LPS and IFN- γ induced PGE₂, NO and TNF- α expressions in murine RAW264.7 cells.

Compounds*	PGE ₂		NO		TNF- α	
	IC ₅₀ (μ M)	95% CI (μ M)	IC ₅₀ (μ M)	95% CI (μ M)	IC ₅₀ (μ M)	95% CI (μ M)
Andrographolide	8.80	7.4 to 10.4	7.4	6.7 to 8.1	23.3	20.1 to 27.0
Diclofenac	~ 0.01	0.001 to 0.1	222	169 to 292	>333	-
Aspirin	14.10	10.1 to 19.7	>1600	-	>1600	-
Paracetamol	7.73	6.14 to 9.73	2763	2406 to 3174	>6000	-
Ibuprofen	0.09	0.08 to 0.11	1058	949 to 1180	839	381 to 1845

255 *All cell viabilities were verified using the MTT assay and were found to be non-toxic at all tested concentrations.

256 **Figure 1.** Dose-response curves of andrographolide compared with ibuprofen, aspirin, diclofenac, and paracetamol in inhibiting LPS and IFN- γ induced PGE₂ (A), NO (B) and TNF-
257 α (C) expressions on RAW264.7 cells. The data has been fitted using a log (inhibitor) vs. normalised response curve with variable slope model ($n = 3$). The error bar expresses the
258 standard error of the mean.

3.2. Broad cytokine-inhibiting effects of andrographolide on LPS stimulated THP-1 cells

The cytokine-inhibiting activity of andrographolide in PMA-differentiated THP-1 cells under the stimulation of LPS was studied. Upon the stimulation of LPS, the cytokines TNF- α , IFN- γ , IL-1 β , IL-2, IL-4, IL-6, G-CSF, GM-CSF and MCP-1 were upregulated significantly ($p < 0.05$), which were captured and quantified through the BioPlex 100 Bio-Plex system. Although several other cytokines were detected, they could not be quantified because their concentrations lay outside the calibration range for the substances of interest, namely, IL-5, IL-7, IL-10, IL-12, and IL-13 (all below the detection limit) and IL-8 and MIP-1b (both higher than the highest standard).

As summarised in **Table 2**, andrographolide exhibited consistent inhibitory effects on multiple cytokines against LPS stimulation, with IC₅₀ values ranging from 12.2 to 65.2 μ M. Most NSAIDs showed little to no activity (IC₅₀ values > 150 μ M). The most potent cytokine-inhibiting activity of andrographolide was seen in IL-6, with an IC₅₀ of 12.2 μ M with a 95% CI range of 9.1 to 16.2 μ M, indicating minimal variation. IL-6 is primarily considered a proinflammatory cytokine, contributing to host defence in response to infections and tissue injury, and its dysregulation is associated with the pathogenesis of chronic inflammation and autoimmunity [51]. Moreover, recent clinical trials have shown that IL-6 plays a central role in the mechanism of the cytokine storm, and serves as a predictor for disease severity and mortality in COVID-19 [52]. Thus, IL-6 and its receptor have been suggested as important therapeutic targets for cytokine storm, and tocilizumab, an IL-6 receptor (IL-6R) antagonist, was repositioned for the trials of cytokine storm against COVID-19 during the pandemic [53]. However, the cytokine-inhibiting activity of andrographolide is not restricted to IL-6 as it also influences other central cytokines.

TNF- α , IFN- γ and IL-1 β contribute to the escalation of the cytokine storm through different modes of action. In influenza viral infection-induced cytokine storm, the reduction of TNF- α results in improved body weight and survival, despite a minimal impact on viral clearance, indicating that it may be a promising therapeutic target [54]. IFN- γ is a potent antiviral cytokine mediated through JAK-STAT pathway. However, its overexpression is linked to lung injury [55]. The role of IL-1 β in cytokine storm is associated with the induction of NLRP3 inflammasome, and its function is quite complex, promoting viral clearance and immune pathology. Our data indicated that andrographolide significantly inhibited TNF- α , IFN- γ and IL-1 β simultaneously, with IC₅₀ values of 29.3, 31.4 and 18.1 μ M, respectively. This result tentatively indicates andrographolide's potent activity in reducing cytokine release *via* different modes of action. In contrast, none of the tested NSAIDs achieved 50% inhibition within the tested concentration range (0-200 μ M), and the IC₅₀ values were either estimated over 150 μ M or out of the range at the upper calibration limit.

Andrographolide also demonstrated inhibitory activity in chemokines, including G-CSF, GM-CSF and MCP-1. Increased levels of these chemokines were also detected in COVID-19 patients who presented with acute respiratory distress syndrome [56]. The functions of these chemokines are associated with the recruitment of macrophages, neutrophils and other polymorphonuclear cells to inflammatory sites, which then escalates into an inflammatory cascade. Therefore, the suppressive effect of andrographolide could be beneficial in preventing inflammatory cascades. It should be noted that andrographolide also inhibited the release of IL-2 (IC₅₀ = 35.7 μ M) and IL-4 (IC₅₀ = 32.8 μ M), which play a vital role in the downregulation of immune responses [57]. IL-2 exerts both immunoregulatory and immunostimulatory activities, which are pivotal for cellular activation, and play an important role in primary T-cell responses and an essential role in secondary T-cell responses [57]. IL-4, a Th2-type cytokine, plays an active role in both the innate and adaptive immune response by suppressing Th1-type responses generated by the production of IFN- γ and TNF- α and inhibiting intracellular killing by macrophages [58]. Thus, the action of andrographolide in reducing IL-2 and IL-4 may not be desirable in the treatment of any cytokine storm and hyperinflammation associated with these cytokines, however, further *in vivo* studies are warranted to examine the overall effect of andrographolide in regulating the whole cascade of the cytokine storm.

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Table 2. The half maximal cytokines inhibitory concentrations of andrographolide, diclofenac, aspirin, paracetamol, and ibuprofen in LPS-induced THP-1 cells.

Cytokines	Andrographolide		Diclofenac		Aspirin		Paracetamol		Ibuprofen	
	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)
TNF-α	29.3	24.7 to 34.7	~469	IF	>1000	IA	>6000	IA	~1671	IA
IFN-γ	~31.4	IF	~162	IF	>1000	IA	>6000	IA	~1574	IA
IL-1β	18.1	5.1 to 63	~151	IF	>1000	IA	~4362	IA	~1241	IA
IL-2	35.7	28.3 to 45.0	~365	IF	>1000	IA	~6000	IA	~1663	IA
IL-4	32.8	28.2 to 38.3	~326	IF	>1000	IA	>6000	2017 to 20101	~1519	IA
IL-6	12.2	9.1 to 16.2	~189	IF	>1000	IA	~920	IF	~907	IF
G-CSF	31.90	22.49 to 45.26	~213	IF	>1000	IA	~6831	3108 to 15012	~1800	IA
GM-CSF	65.2	31.5 to 135.0	~405	IF	>1000	IA	>6000	IA	>1500	IA
MCP-1	45.95	26.41 to 79.96	~314	IF	>1000	IA	~2936	IF	~872	IF

(~) - Estimate IC₅₀ due to poor curve fit

IA - Insufficient activity to estimate an IC₅₀ range

IF - Insufficient curve fit to estimate an IC₅₀ range (high hillslope)

3.3. The inhibitory effect of andrographolide on LPS induced NFκB activation

Andrographolide is known to exert anti-inflammatory activity *via* the down-regulation of NFκB activation. While transfecting the cell line with multiple copies of NFκB to study other promoter elements of E-selection in ELAM9 can be interesting, it is beyond the scope of this study. The primary aim of this study was to directly compare the broad-based inhibition of plant-derived andrographolide and other NSAIDs against the cytokines involved in the NFκB inflammatory cascade, prior to any potential clinical application against the cytokine storm [59].

In this study, the activity of andrographolide was compared to that of NSAIDs using flow cytometry on ELAM9-RAW264.7 cells with NFκB green fluorescent protein (GFP). Upon lipopolysaccharide (LPS) stimulation, the ELAM9-RAW264.7 cells expressed NFκB GFP, which was then captured by the flow cytometer. As shown in **Table 3** and **Figure 2**, andrographolide inhibited NFκB activation in a dose-dependent manner with IC₅₀ at 26.0 μM. Most of the tested NSAIDs did not impact NFκB, except for diclofenac which indicated an inhibitory trend (IC₅₀ at 508.3 μM).

Andrographolide was markedly more potent than the NSAIDs in downregulating NFκB activation. NFκB plays a central role in maintaining normal cell function and producing inflammatory mediators in inflammatory conditions and acts as an upstream proinflammatory mediator. The NFκB-mediated signaling pathway interacts with cytokines, including IFNs, and cell survival. The NFκB pathway is often triggered by toll-like receptors upon exposure to viral pathogens, leading to host immune responses and the release of proinflammatory cytokines. It has been recently recognised that COVID-19 activates the NFκB pathway, like MERS and SARS-COV [60], leading to an increased level of inflammatory mediators. The inhibition of NFκB improved the survival of BALB/c mice and reduced SARS-COV-induced inflammation without influencing viral titers [61]. Thus, the potent and broad cytokine-inhibiting effect of andrographolide may be attributed to its down-regulation of NFκB activation.

Table 3: The IC₅₀ values of andrographolide, diclofenac, aspirin, paracetamol and ibuprofen in inhibiting ELAM9-RAW264.7 cells with NFκB green fluorescent protein (GFP).

Assay	Andrographolide		Diclofenac		Aspirin		Paracetamol		Ibuprofen	
	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)	IC ₅₀ (μM)	95% CI (μM)
NF-κB activation IC ₅₀	26.0	23.4 to 29.0	508.3	400.3 to 645.4	>1600	-	>6000	-	>1500	-

Figure 2. Dose-response curves of andrographolide compared with ibuprofen, aspirin, diclofenac, and paracetamol in inhibiting NFκB (GFP) expressions on ELAM9-RAW264.7 cells measured by flow cytometry. Error bars express standard error of the mean and $n = 3$.

In contrast, NSAIDs' defined mechanism of action simplifies the understanding and rationalisation of their anti-inflammatory effect. Their expressed side effects are easily linked to their mechanism of action because it is well understood. Andrographolide's widespread action makes its safety and any potential side effects harder to predict. However, *A. paniculata* has been used for thousands of years in Ayurvedic medicine and is considered safe [62-66]. Andrographolide has been shown to have a high therapeutic index, with a safety margin (LD₅₀) for administration intraperitoneally (11.46 g/kg) [67]. Andrographolide has also been reported to exhibit gastro-protective and ulcer-preventive effects, which, in combination with its anti-inflammatory effects, could potentially make it a safer alternative to traditional NSAIDs [35].

Although this study highlights the promising potential of andrographolide compared to the widely used NSAIDs to address cytokine storm, it is limited as only cell models were studied. Cytokine storm is a complex inflammatory event that is better represented in *in vitro* models and clinical settings. However, these results demonstrate its clinical potential. The *in vitro* results also fail to compensate for the differing pharmacokinetic profiles of each drug, so direct comparison is complicated. It should also be noted that there are several other plant-derived drugs such as resveratrol, tetrahydrocannabinol (THC) and cannabidiol (CBD) have shown promising cytokine-inhibiting activity against cytokine storm in recent studies when used as an adjuvant in COVID-19 treatment [68-71]. Future work could involve studies evaluating the efficacy of these drugs compared to andrographolide against the cytokine storm in a clinical context.

In summary, andrographolide is a promising candidate as an alternative to NSAIDs for broad inflammatory ailments and the management of cytokine storm. However, further *in vivo* and human clinical studies are needed, along with a comprehensive multi-omics understanding of the current findings to confirm its potential efficacy.

4. Conclusion

Andrographolide was shown to possess potent inhibitory effects in LPS and IFN-γ induced PGE₂, NO and TNF-α in the RAW264.7 cells and LPS-induced multiple cytokines in the THP-1 cells, including TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, G-CSF, GM-CSF and MCP-1. The NO and cytokine-inhibitory properties of andrographolide were generally more potent than the common NSAIDs tested in this study (aspirin, ibuprofen, paracetamol and diclofenac). NSAIDs only exhibited higher potency in inhibiting PGE₂, where the activity of andrographolide was comparable to that of paracetamol. The broader cytokine-inhibiting activity of andrographolide was associated with the downregulation of the activation of NF-κB as tested in ELAM9 RAW264.7 cells. Overall, andrographolide may serve as a promising candidate therapeutic compound in regulating multiple cytokines and their associated inflammatory responses. Andrographolide has a broader anti-inflammatory effect than NSAIDs and may be better suited to the complex nature of the inflammatory immune response. Further *in vivo* and human clinical studies are needed to confirm and verify the andrographolide's potential efficacy and side-effect profile.

Supplementary Files: 1. MTT data supplement: "Supplement No.1 MTT Data.zip"; 2. Cell viability & Flow cytometry data: "Supplement No.2 Flow Cytometry.zip".

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away before this research could be published. He made a significant contribution to the research presented in this paper. He will be 384
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Conflicts of Interest: The authors declare no conflict of interest. 388

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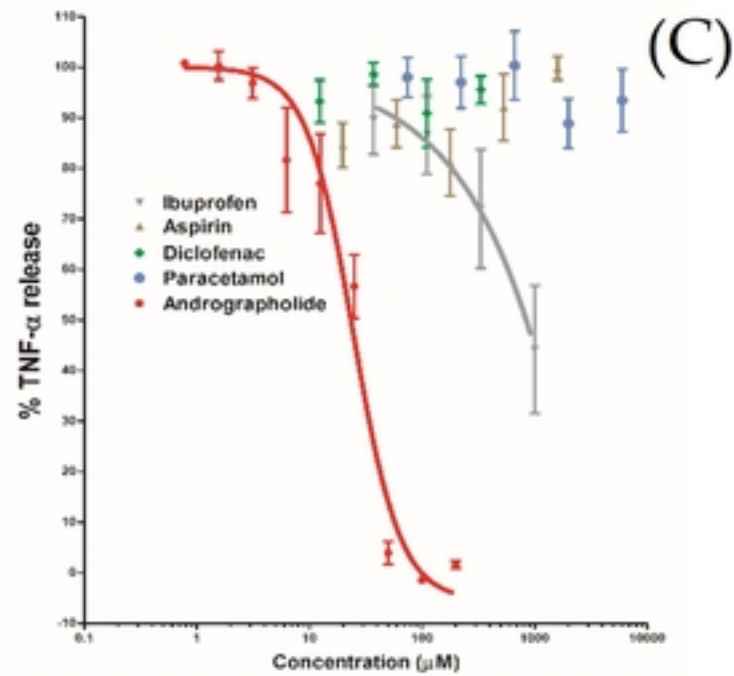
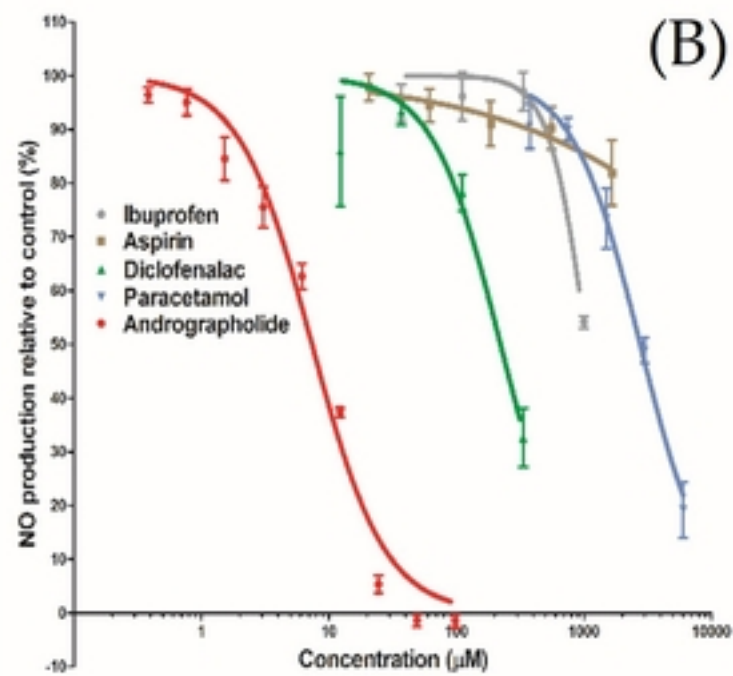
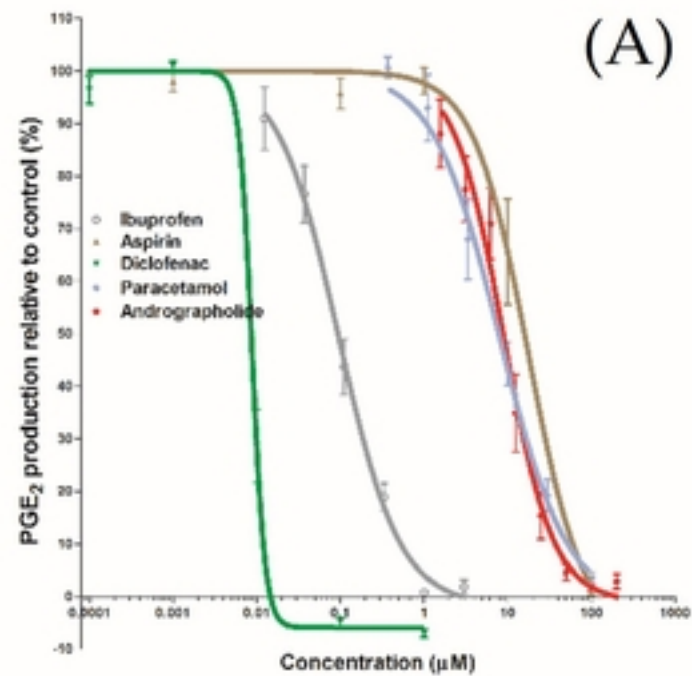


Figure 1

NF- κ B activation assay in ELAM9 RAW264.7 cells

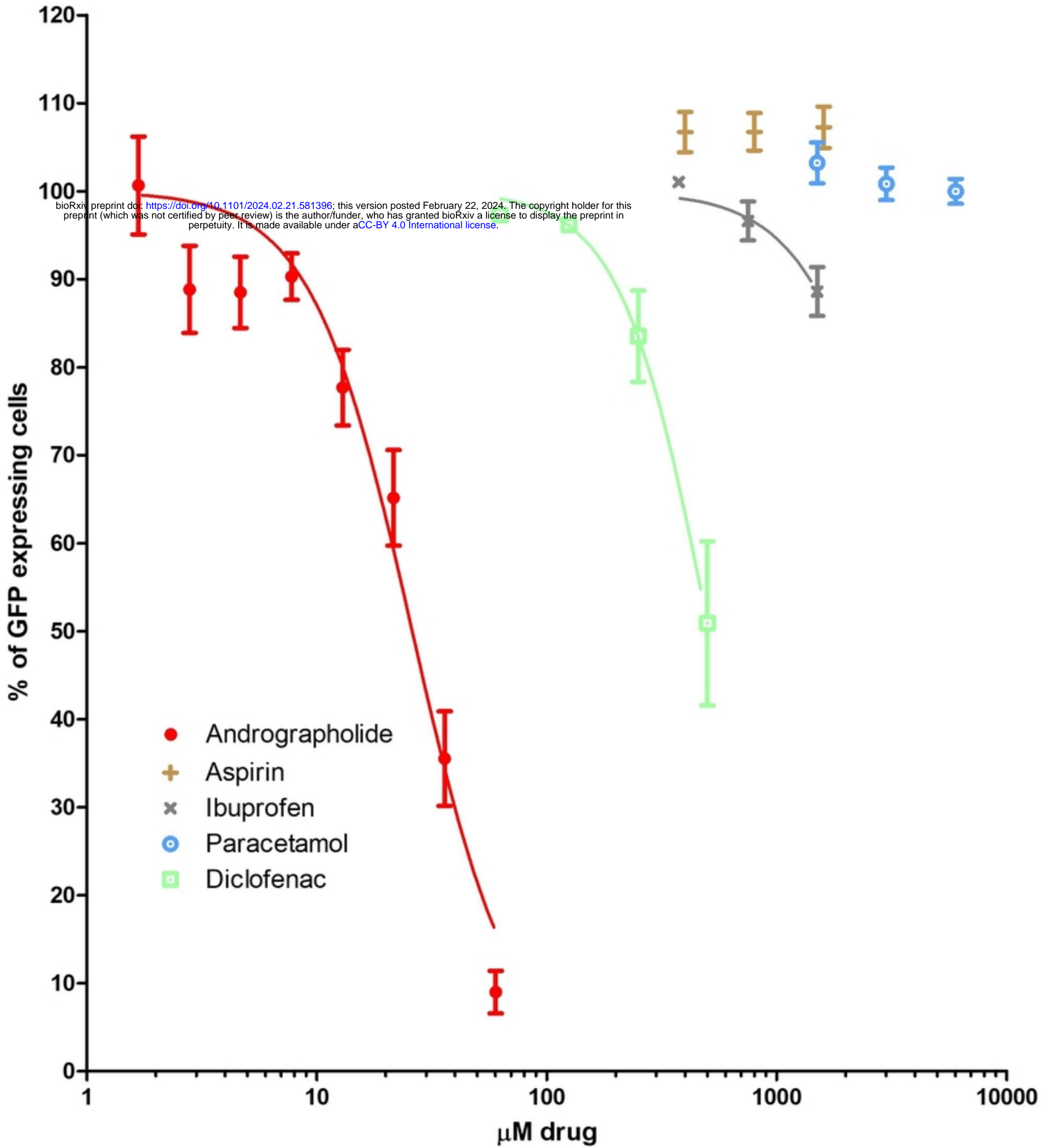


Figure 2