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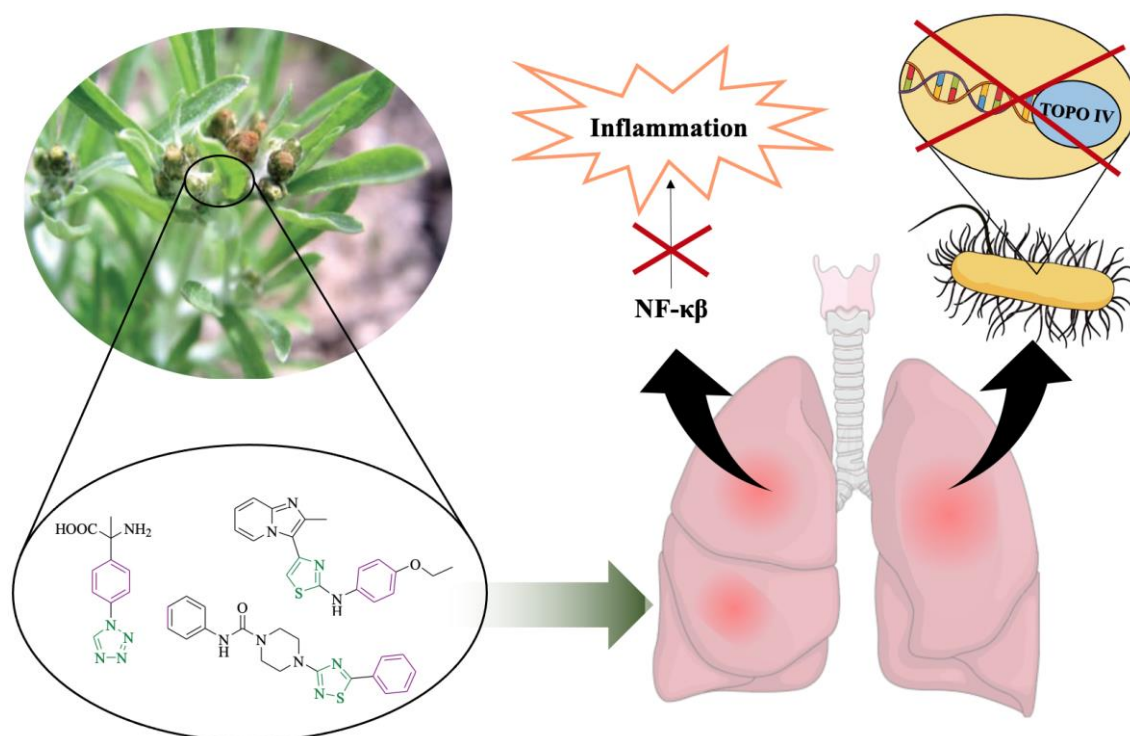
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**Isolation and characterisation of antibacterial and anti-inflammatory compounds
from *Gnaphalium polycaulon***



Isolation and characterisation of antibacterial and anti-inflammatory compounds from
Gnaphalium polycaulon

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Abstract

Ethno-pharmacological relevance: *Gnaphalium polycaulon* commonly known as "cudweed" has been used throughout South America as an infusion to treat colds, bronchitis, fever or pneumonia.

Aim of the study: This study aimed to determine the antibacterial and anti-inflammatory activities of the aqueous extract of *Gnaphalium polycaulon* and identify the related compounds.

Materials and methods: A bio-guided isolation of the active compounds of *Gnaphalium polycaulon* was carried out, selecting the fractions depending on their antibacterial, anti-inflammatory and cytotoxic activities. The antibacterial effect was studied against *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Streptococcus pneumoniae*; and the anti-inflammatory study was performed by measuring the inhibition of NF- κ B in BEAS-2B and IMR-90 cell cultures.

Results: Three compounds were obtained and characterised by nuclear magnetic resonance and mass spectrometry. These compounds are 2-(4-(1-H-tetrazol-1-yl) phenyl)-2-aminopropanoic acid (**1**), *N*-phenyl-4-(3-phenyl-1,2,4-thiadiazol-5-yl) piperazine-1-carboxamide (**2**) and *N*-(4-ethoxyphenyl)-4-(2-methylimidazo-[1,2- α] pyridine-3-yl) thiazol-2-amine (**3**). All compounds showed antibacterial activity with MIC values of 44.80-44.85, 0.017-0.021 and 0.0077-0.0079 μ M, respectively, in the *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Streptococcus pneumoniae* strains, while the positive control, Ofloxacin, had a MIC value of 27.64-27.67 μ M. This was corroborated through a zone inhibition assay, where compound **3** (11.36-11.67 mm) was much more active than the positive control (Ofloxacin, 23.41-24.12 mm), while compounds **2** (26.47-27.64 mm) and **1** (28.39-29.76 mm) displayed similar antibacterial potential to the positive control. Finally, all the compounds presented NF- κ B inhibitory activity, compounds **3** (IC₅₀=0.0071-0.0073 μ M) and **2** (IC₅₀=0.016-0.019 μ M) being the most promising. Compound **1** (IC₅₀=44.24-44.26 μ M) had less anti-inflammatory

44 potential, being also the closest to the values displayed by the positive control (Celastrol,
45 $IC_{50}=7.41\ \mu M$).

46 *Conclusion:* In the present study, three compounds were isolated for the first time from the
47 aqueous extract of *Gnaphalium polycaulon*. Their antibacterial and anti-inflammatory potential
48 was tested and showcased.

49

50 **Keywords:** *Gnaphalium polycaulon*; antibacterial; anti-inflammatory; respiratory tract
51 infection

52 **Abbreviations:**

53	AcOEt:	Ethyl acetate
54	ACT:	Actinomycin D
55	ATCC:	American Type Culture Collection
56	BEAS-2B	Human lung and bronchus epithelial cells
57	BOD:	Bio-oxygen demand
58	CAP:	Community-acquired pneumonia
59	CC ₅₀ :	Cytotoxic concentration 50%
60	CFU:	Colony-forming unit
61	DCM:	Dichloromethane
62	DH ₂ O:	Distilled water
63	DMEM:	Dulbecco's modified eagle medium
64	DMSO:	Dimethyl sulfoxide
65	DTT:	Dithiothreitol
66	ECDC	European Centre for Disease Prevention and Control
67	ELISA:	Enzyme-linked immunosorbent assay
68	Et ₂ O:	Diethyl ether
69	EtOH:	Ethanol
70	FBS:	Fetal bovine serum
71	HEX:	<i>n</i> -hexane
72	IC ₅₀ :	Inhibitory concentration 50%
73	IL-6	Interleukin-6
74	IL-8	Interleukin-8
75	IMR-90	Human lung fibroblasts
76	LDH:	Lactate dehydrogenase

77	MeOH:	Methanol
78	MHB:	Mueller-Hinton broth
79	MIC:	Minimum inhibitory concentration
80	MMP-3	Matrix metalloproteinase-3
81	NBM:	Nutrient broth medium
82	NF- κ B:	Nuclear factor kappa-light-chain-enhancer of activated B cells
83	PBS:	Phosphate-buffered saline
84	QTOF:	Quadrupole time-of-flight
85	RLUs:	Relative luminescence units
86	RTI:	Respiratory tract infections
87	TLC:	Thin layer chromatography
88	TMS:	Tetramethylsilane
89	TNF- α :	Tumour necrosis factor- α
90	ZI:	Zone of inhibition
91		

1. Introduction

The lower respiratory tract is vulnerable to infections caused by a wide variety of microorganisms, because it is one of the systems that directly links the human body to the environment (Singh et al., 2020). If the right circumstances are present and host factors allow, practically any microorganism can cause lower respiratory tract infection (Mahowald et al., 2019).

In this context, bacteria are one type of microorganisms, that represent a common cause of respiratory infection (Prat & Lacoma, 2016). For example, *Streptococcus pneumoniae* (*S. pneumoniae*) is the most common pathogen that causes community-acquired pneumonia (CAP) worldwide, accounting for 15% of pneumonia cases in the United States and 27% of cases worldwide in today (Dion & Ashurst, 2021). Likewise, *Staphylococcus aureus* (*S. aureus*) it is one of the main commensal and opportunistic pathogens responsible for causing lower respiratory tract infections (RTI). The prevalence of colonisation by these bacteria makes it difficult to establish an adequate treatment (McGuire et al., 2020). Moreover, according to data from the European Centre for Disease Prevention and Control (ECDC), 3379 confirmed cases of invasive *Haemophilus influenzae* (*H. influenzae*) disease was reported in Europe in 2016, with a rate of 0.7 cases per 100.000 population (Khattak & Anjum, 2021). Finally, RTI caused by *Moraxella catarrhalis* (*M. catarrhalis*) occur in individuals over 50 years of age, being considered a primary pathogen (Hirai et al., 2020).

On the other hand, a common consequence of a bacterial infection is an inflammatory response coordinated by the activation of signalling pathways that regulate the levels of inflammatory mediators in resident tissue cells and inflammatory cells recruited from the blood (Aghasafari et al., 2019). The nuclear factor kappa-activated B-cell enhancer-light chain (NF- κ B) is a central inflammatory mediator that is essential for the induction of a variety of inflammatory genes in response to various pathogens and inflammatory cytokines (Rahman &

McFadden, 2011). One of these cytokines is the tumour necrosis factor- α (TNF- α), a key regulator of host responses to infection, particularly immune responses to bacterial infections (Wang et al., 2020).

However, it has been shown that a prolonged inflammatory response can damage tissues, so inhibition of inflammatory mediators (NF- κ B) would reduce infection (Pahwa et al., 2020). One of the main complications of a bacterial infection is the inflammation of the respiratory tract (Varricchio et al., 2020), leading to a narrowing of the airways, and sometimes, even difficulties with breathing. Generally, cases of tracheitis, tracheobronchitis and bronchitis are complicated by an infection of microbial nature, and frequently, wide spectrum antibiotics (Alzahrani et al., 2018) are administrated in very long therapies (Leekha et al., 2011).

Although there are a variety of broad-spectrum antibiotics, bacteria have developed resistance to them, which means that they can develop tolerance to specific concentrations, leading to the need to increase such concentrations if the effect is to be achieved again (Breijyeh et al., 2020). The failure of these treatments has generated the need to search for new antibacterial compounds in plant extracts, an alternative enabling the obtention of new drugs against bacteria (Kokoska et al., 2019).

Medicinal plants are traditionally used throughout the world as remedies for the treatment of various diseases, including respiratory problems due to the particular nature of their compounds (Dias et al., 2012). These compounds have demonstrated beneficial advantages in terms of antioxidant, anti-inflammatory and antibacterial activities (Guglielmi et al., 2020). They can restore the clinical application of older antibiotics by increasing their potency and, therefore, avoid the development of drug resistance (Browne et al., 2020). Based on their chemical structures, they can be classified into several major groups that include alkaloids, sulphur-containing compounds, terpenoids, and polyphenols (Radulović et al., 2013).

In this context, *Gnaphalium polycaulon* Pers. (*G. polycaulon*), commonly known as “Cudweed”, *G. floccosum*, *G. gracillimum*, *G. multicaule*, *G. niliacum* and *G. schomburgkii* (The Plant List, 2021) belonging to the Asteraceae family is traditionally used in form of infusions for the treatment of colds, flu, tonsillitis, laryngitis, pneumonia and congestion and many types of infections (Uniyal & Shiva, 2005; Kaminidevi et al., 2015).

Only three 3-hydroxydihydrobenzofuran glucosides have been isolated from the aqueous extract of *G. polycaulon* (Sahakitpichan et al., 2011). However, flavonoids (Torrenegra et al., 2018), sesquiterpenes, diterpenes, triterpenes, phytosterols, anthraquinones and caffeoylquinic acid derivatives were isolated in other species of this genus (Zheng et al., 2013); showing interesting pharmacological activities. Among these pharmacological properties, antibacterial, antifungal, antitussive, expectorant and anti-inflammatory (Zheng et al., 2013) activities stand out.

The objective of our study is to validate the traditional uses of the aqueous extract from *G. polycaulon* through the bio-guided separation of its compounds with antibacterial and anti-inflammatory activities.

2. Material and methods

2.1. Plant Material

The aerial parts of *Gnaphalium polycaulon* were collected from the San Juan Huancollo community, Ingavi province, La Paz, Bolivia (16°35'15.1"S 68°58'21.2"W), in June 2019, at an altitude of 3918 m. The botanical identification was confirmed by the National Herbarium of Bolivia (No. 16055).

2.2. General experimental procedures

Column chromatography was performed with silica gel (20-45 μm and 40-63 μm , Merck). Thin layer chromatography (TLC, Merck Silica gel 60-F₂₅₄ plates) was used for compounds identification. TLC plates thus obtained were visualised by UV light (Spectroline® E-Series UV lamp with one longwave (365 nm) and one shortwave (254 nm) tube, 230V, New York, USA) and through heating a plate stained with a 5% H₃PMo₁₂O₄₀ (Phosphomolybdic acid) solution in ethanol (EtOH) 95% followed by heat application. All organic solvents that were used for isolating the compounds were purchased from Sigma-Aldrich.

NMR experiments were performed on the Bruker Advance DRX 300 and 500 spectrometers operating at 300 MHz, 500 MHz (¹H) or 75 MHz, and 126 MHz (¹³C) with tetramethylsilane (TMS) as the reference solvent (δ_{H} 0 ppm). Spectra were calibrated by assignment of the residual solvent peak to δ_{H} 7.26, δ_{H} 3.31 and δ_{C} 77.16, δ_{C} 49.00, for CDCl₃ and MeOD, respectively. The complete assignment of protons and carbons was done through ¹H-¹H COSY, HSQC and HMBC spectra analysis.

HREIMS analyses were performed using a mass spectrometer with a hybrid quadrupole time-of-flight (QTOF) analyser model MAXIS II from the commercial house Bruker, S.A. Samples were analysed using the electrospray ionisation technique, by direct infusion at a flow of 3 $\mu\text{L}/\text{min}$, using methanol with 0.1% formic acid as the ionising phase. The source parameters were the following: End Plate Offset: 500 V; Capillary: 3500; Nebulizer: 0.2 bar; Dry Gas: 2.0 l/min; Dry Temp.: 250°C; and Mass range of 50-3000 Da.

2.3. Extraction and isolation

The air-dried leaves (1 kg) from *G. polycaulon* were extracted by decoction (30 min at boiling point) with 2 L distilled water (DH₂O). The resulting aqueous extract was frozen in glass containers at a temperature of -38°C and then lyophilised (Freeze dryer, Christ alpha 1e2

LD plus, Germany) at -50°C. Subsequently, the residue (300 g) was further extracted with *n*-hexane (HEX, 3x400 mL), dichloromethane (DCM, 3x400 mL), methanol (MeOH, 3x400 mL). Subsequently, the sub-extracts were filtered, and the respective solvents were removed by vacuum rotary evaporation at room temperature (25°C). As a result, four extracts of 1.8 g, 1.2 g, and 0.6 g, respectively, were obtained.

The dichloromethane sub-extract was selected as the most active and less cytotoxic, so it was fractionated using a chromatographic column (2x50 cm), employing Si-60 silica gel (40-63 μ m, Merck) as the stationary phase, and using a gradual gradient of HEX/Ethyl acetate (AcOEt) (5:1-3:1 v/v) as eluent. Eight fractions were obtained: **F1** (120 mg), **F2** (25 mg), **F3** (200 mg), **F4** (5.2 mg), **F5** (200 mg), **F6** (70 mg), **F7** (2.6 mg) and **F8** (1.5 mg).

Subsequently, based on the biological activity data, separations of **F1**, **F3** and **F6** fractions were carried out. Fraction **F1** (120 mg) was separated employing a chromatography column (2x50 cm) with Si-60 Silica gel (40-63 μ m, Merck) as a stationary phase and a gradual gradient of HEX/AcOEt (2:1-1:1 v/v) as eluent. Three sub-fractions were obtained: **F1a** (17.2 mg), **F1b** (3.2 mg) and **F1c** (19.5 mg). Subsequently, based on the pharmacological activity assays, the **F1b** sub-fraction was purified using a microcolumn, obtaining compound **1** (3.2 mg).

Subsequently, fraction **F3** (200 mg) was columned employing a chromatography column (2x50 cm) with Si-60 Silica gel (40-63 μ m, Merck) as a stationary phase and a gradual gradient of HEX/ Diethyl ether (Et₂O) (1:2 v/v) as eluent. It led to obtaining five sub-fractions: **F3a** (21.7 mg), **F3b** (6.1 mg), **F3c** (55.5 mg), **F3d** (30 mg) and **F3e** (70 mg). Based on the pharmacological activity potential of each sub-fraction, the **F3d** sub-fraction was purified employing a microcolumn, obtaining compound **2** (5.4 mg).

Finally, seven sub-fractions (**F6a** 8 mg; **F6b** 3 mg; **F6c** 3.1 mg; **F6d** 4.0 mg; **F6e** 3.1 mg; **F6f** 3.2 mg and **F6g** 6.7 mg) were obtained from the separation of **F6** (70 mg) employing a

chromatography column (2x50 cm) with Si-60 Silica gel (40-63 μm , Merck) as a stationary phase and a gradual gradient of HEX/AcOEt (2:1 v/v) as eluent. Analysing the results of the pharmacological activity assays, the **F6c** sub-fraction that presented the highest activity was purified by means of a microcolumn, obtaining compound **3** (2.1 mg).

2.4. *In-vitro* antibacterial activity

2.4.1. *Bacteria*

In the present study, strains of four bacteria were used: *H. influenzae* (*Haemophilus influenzae*, 33391), *M. catarrhalis* (*Moraxella catarrhalis*, BAA-1424), *S. aureus* (*Staphylococcus aureus*, BAA-3113) and *S. pneumoniae* (*Streptococcus pneumoniae*, BAA-2298) obtained from the American Type Culture Collection (ATCC).

2.4.2. *Broth microdilution method (MIC)*

The minimum inhibitory concentration (MIC) of the samples against the bacterial strains was determined using the microdilution method in 96 well plates (Cellstar®, Greinerbio-one, Germany) (Apaza et al., 2021). The Mueller-Hinton broth (MHB) medium (180 μL) of the bacterial culture was used to fill the first experimental well. The other wells were filled with 100 μL of medium. Subsequently, a volume of 20 μL of extracts, sub-extracts, fractions, sub-fractions, and compounds was added to the first well. Double fold serial dilution was then carried out in the same plate. Overnight batch culture of the bacteria (10 μL) was used to inoculate each well until a size of ca. 1×10^6 Colony-forming unit (CFU)/mL was achieved. The plates were incubated for 48 h at 37°C. MICs values were calculated following the process used by Apaza et al. (2021). Dimethyl sulfoxide (DMSO) at the same tested concentration was used as a negative control, while Ofloxacin (Sigma-Aldrich, CAS

Number 82419-36-1) was used as a positive control (of 10.01 $\mu\text{g/mL}$, equivalent to 27.67 μM for the compounds) to assess the accuracy of the MIC method. Each MIC determination was carried out in triplicate.

2.4.3. Agar well diffusion method

For the cultivation of the bacterial strains, nutrient broth medium (NBM) was prepared using 8% nutrient broth in double DH_2O and agar-agar, for 30 min/s at 103.42 kPa. Agar plates were prepared by pouring 15 mL of NBM into petri dishes under aseptic condition and kept at 25°C for stabilisation. Bacterial cell cultures were maintained in peptone saline solution by regular sub-culturing at 37°C for 24 h (Apaza et al., 2021).

Agar plates were inoculated by streaking 3 times the swab of bacterial strains over the entire sterile agar surface, and rotating the agar plate at 60° for uniform distribution of the inoculum. The plates were dried at 25°C under aseptic condition followed by boring of 9 mm diameter wells. Serial dilutions (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 and 0.2 $\mu\text{g/mL}$) of the samples (extract, sub-extracts, fractions, sub-fractions and compounds) were prepared with DMSO, using Ofloxacin (Sigma-Aldrich, CAS Number 82419-36-1) as a positive control at a concentration of 10.01 $\mu\text{g/mL}$ (equivalent to 27.67 μM for the compounds). The samples (100 μL) were added in wells using a sterile micropipette. Subsequently, the plates were incubated in a bio-oxygen demand (BOD) incubator at 37°C for 48 h. The zone of inhibition (ZI) of each bacterial strain was measured with a calibrated digital Vernier caliper in triplicate.

2.5. Cell culture reagents and drugs

Two cell lines were used in this study: BEAS-2B (Human lung and bronchus epithelial cells, ATCC CRL-9609) and IMR-90 (Human lung fibroblasts, ATCC CCL-186) cells. All cell

lines were obtained and cultured in specific media according to ATCC, an atmosphere of 95% air and 5% CO₂ at 37°C.

Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Summit Biotechnology; Ft. Collins, CO) and phosphate-buffered saline (PBS, SAFC Biosciences, Inc. Andover-Hampshire, UK) was used as a culture medium. L-glutamine was obtained from Applichem. Penicillin (100 U/mL) and Streptomycin (100 µg/mL) were purchased from Fisher Scientific (Pittsburgh, PA). The extracts, sub-extracts, fractions and sub-fractions dissolved in 20 mg/mL of DMSO, and the compounds in 10 mM of DMSO.

2.6. Cytotoxicity assay

The samples with different concentrations (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 and 0.2) in µg/mL (extract, sub-extracts, fractions and sub-fractions) or µM (compounds) were analysed in a panel of two cell lines (BEAS-2B and IMR-90) by means of the lactate dehydrogenase (LDH) assay. The cells (3x10³ cells/well) were seeded in 96-well plates and incubated overnight at 37°C in a humidified atmosphere of 5% CO₂. Afterwards, the cells were treated with the samples obtained at different concentrations and using DMSO as a control for 48 h. Actinomycin D (ACT ≥95% Sigma-Aldrich; CAS Number 50-76-0) was used as a positive control at a concentration of 0.01 µg/mL (equivalent to 0.008 µM for the compounds). When 48 h of treatment had passed, the samples were put in a LDH kit (Innoprot Company), and culture supernatants (100 µL) were collected and incubated in the reaction mixture. After 30 min, the reaction was stopped by the addition of 1 M HCl, and the absorbance (490 nm) was measured using a spectrophotometric enzyme-linked immunosorbent assay (ELISA plate reader, SpectraMax® i3, Molecular Devices).

2.7. *In vitro* anti-inflammatory activity (NF- κ B inhibition assay)

In order to determine the inhibition potential of NF- κ B, the protocol described by Apaza et al. (2021) was used. The cells (3×10^3 cells/well) were seeded the day before the assay on a 96-well plate and then treated with the samples (extract, sub-extracts, fractions, sub-fractions and compounds) at the same concentrations used in the viability assays for 15 min and then they were stimulated with 30 ng/mL of TNF- α . Celastrol ($\geq 98\%$ Sigma-Aldrich, CAS Number 34157-83-0) was used as a positive control at a concentration of 3.34 μ g/mL (equivalent to 7.41 μ M for the compounds). When 48 h had passed, the cells were washed twice with PBS and lysed for 15 min in a 50 μ L buffer containing 25 mM $\text{C}_4\text{H}_{11}\text{NO}_3 \cdot \text{H}_3\text{PO}_4$ (Tris-phosphate, pH=7.8), 8 mM MgCl_2 , 1 mM dithiothreitol (DTT), 1% Triton X-100 and 7% glycerol, at room temperature, using a horizontal shaker. The luciferase activity was measured with a GloMax 96 microplate luminometer (Promega) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The relative luminescence units (RLUs) and the results were calculated and expressed as the percentage of inhibition of NF- κ B activity induced by TNF- α (100% activation). The experiments for each concentration used the assay were performed in triplicate.

2.8. *Statistical analysis*

Cytotoxic concentration 50% (CC_{50}) and inhibitory concentration 50% (IC_{50}) values were determined by non-linear regression. All the experiments were performed in triplicate. One-way ANOVA statistical analysis (Tukey's multiple comparisons test, $**p < 0.05$; $***p < 0.001$) was performed to evaluate if the differences among values are statistically significant. All the analyses were performed using 1994-2020 GraphPad Prism Software LLC version 9.0.0. (86) for Mac (producer Dennis Radushev, San Diego, California USA, www.graphpad.com).

3. Results

3.1. Extraction, isolation and characterisation of compounds

In this report we have included the characterisation of three compounds (**1**, **2** and **3**) that have been previously reported as synthesised compounds; however, this is the first time they are isolated as natural compounds (Figure 1).

Figure 1.

3.1.1. Characterisation of compound 1

2-(4-(1-H-tetrazol-1-yl)-phenyl)-2-aminopropanoic acid (**1**) was obtained as a white amorphous powder; $R_f=0.68$ (HEX/AcOEt 1:2); ^1H NMR (300 MHz, MeOD) δ_{H} 8.11, (d, $J=8.6$ Hz, 2H/H-3', H-5'), 7.78, (d, $J=8.6$ Hz, 2H/H-2', H-6'), 1.97 (s, 3H/H-3); ^{13}C NMR (75 MHz, MeOD) δ_{C} 174.4 (C-1), 158.4 (C-5'''), 142.5 (C-1'), 128.5 (C-3', C-5'), 128.1 (C-2', C-6'), 127.3 (C-4'), 63.7 (C-2), 22.7 (C-3); HRESIMS m/z 234.10 $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{10}\text{H}_{12}\text{N}_5\text{O}_2$, and 255.07 $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{10}\text{H}_{11}\text{N}_5\text{O}_2\text{Na}$. The spectroscopic data obtained for compound **1** was corroborated with the available literature references (Jane et al., 1995).

3.1.2. Characterisation of compound 2

N-phenyl-4-(3-phenyl-1,2,4-thiadiazol-5-yl)piperazine-1-carboxamide (**2**) was obtained as a white amorphous powder; $R_f=0.35$ (HEX/AcOEt 9:2); ^1H NMR (300 MHz, CDCl_3) δ_{H} 8.22-8.18 (m, 2H/H-2', H-6'), 7.46-7.41 (m, 3H/H-3', H-4', H-5'), 7.38-7.30 (m, 4H/H-2'', H-3'', H-5'', H-6''), 7.09 (t, $J=7.0$ Hz, 1H/H-4'''), 3.72 (s, 8H/H-2'', H-3'', H-5'', H-6''); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} 185.3 (C-5), 170.4 (C-3), 155.0 (C-7''), 138.6 (C-1'''), 133.4 (C-1'), 130.2

(C-4'), 129.2 (C-3', C-5'), 128.6 (C-3'', C-5''), 128.2 (C-2', 6'), 123.9 (C-4''), 120.3 (C-2'', C-6''), 48.5 (C-3'', C-5''), 43.5 (C-2'', C-6''); HRESIMS m/z 366.14 $[M+H]^+$ calculated for $C_{19}H_{20}N_5OS$ and 388.12 $[M+Na]^+$ calculated for $C_{19}H_{19}N_5OSNa$. The spectroscopic data obtained for compound **2** was corroborated with the available literature references (Keith et al., 2008).

3.1.3. Characterisation of compound 3

N-(4-ethoxyphenyl)-4-(2-methylimidazo-[1,2- α]pyridine-3-yl)thiazol-2-amine (**3**) was obtained as a white amorphous powder; R_f =0.50 (HEX/AcOEt 1:2); 1H NMR (500 MHz, $CDCl_3$) δ_H 9.18 (d, J =6.9 Hz, 1H/H-5''), 8.18-8.12 (m, 1H/H-8''), 7.65 (t, J =7.9 Hz, 1H/H-7''), 7.40 (dd, J =2.4, 6.4 Hz, 2H/H-2', H-6'), 7.22 (t, J =6.9 Hz, 1H/H-5), 6.93 (dd, J =2.4, 6.4 Hz, 2H/H-3', H-5'), 6.71 (s, 1H/H-6''), 4.09- 4.00 (m, 2H/H-7'), 2.72 (bs, 3H/H-10''), 1.45-1.42 (m, 3H/H-8'); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 168.4 (C-2), 156.6 (C-4'), 138.7 (C-2''), 132.9 (C-1'), 131.6 (C-3''), 127.8 (C-5'', C-7''), 122.7 (C-2'), 116.5 (C-8''), 115.6 (C-3', C-5'), 113.3 (C-6''), 108.0 (C-5), 64.0 (C-7'), 15.0 (C-10''), 11.6 (C-8'); HRESIMS m/z 351.13 $[M+H]^+$ calculated for $C_{19}H_{19}N_4OS$. The spectroscopic data obtained for compound **3** was corroborated with the available literature references (Lee et al., 2007).

3.2. Antibacterial activities of the extracts

MIC and ZI experiments of *G. polycaulon* extract and sub-extracts were carried out against *H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pneumoniae*. The dichloromethane sub-extract (IC_{50} =20.32-20.90 $\mu g/mL$) showed the most promising antibacterial effect through the MIC assay with a statistically significant difference ($p<0.001$) when compared with the aqueous extract and other sub-extracts of *G. polycaulon* (Table 1). Nevertheless, the effect was smaller than the one of the positive control (Ofloxacin, IC_{50} =10.01 $\mu g/mL$, $p<0.001$).

Table 1.

On the other hand, the ZI results suggested that the dichloromethane sub-extract (10.06-10.94 mm) had a statistically significant higher antibacterial effect than the aqueous extract ($p<0.001$), the other sub-extracts of *G. polycaulon* ($p<0.001$), and the positive control (Ofloxacin, 27.12-27.89 mm) ($p<0.001$) (Table 2).

Table 2.

3.3. Cytotoxic activity of the extract and sub-extracts

Regarding the cytotoxicity assay of the extract and sub-extracts of *G. polycaulon*, the results show that the aqueous extract ($CC_{50}=92.88-93.68 \mu\text{g/mL}$) and the sub-extract of dichloromethane ($CC_{50}=86.49-86.98 \mu\text{g/mL}$) presented statistically significant lower cytotoxicity in the BEAS-2B and IMR-90 cell lines than the positive control (ACT, $CC_{50}=0.01 \mu\text{g/mL}$) ($p<0.001$) (Table 3).

Table 3.

3.4. Anti-inflammatory activities of the extract and sub-extracts

In relation to the anti-inflammatory effect, the results showed that the dichloromethane sub-extract ($IC_{50}=20.03-21.71 \mu\text{g/mL}$) presented a statistically significant higher inhibition of the NF- κ B production ($p<0.001$) in all cell lines than the other sub-extracts, followed by the aqueous extract ($IC_{50}=42.77-42.92 \mu\text{g/mL}$) ($p<0.001$). However, the dichloromethane sub-extract and aqueous extract had statistically significant lower inhibition potential than the positive control (Celastrol, $IC_{50}=3.32-3.34 \mu\text{g/mL}$) ($p<0.001$) (Table 4).

Table 4.

Based on the activity results of the sub-extracts, the dichloromethane sub-extract was fractionated using a gradual gradient of HEX/AcOEt (5:1-3:1 v/v) as eluent, from where eight fractions were obtained. These fractions were subjected to antibacterial, cytotoxicity and anti-inflammatory assays.

3.5. Antibacterial activities of the fractions

Regarding the antibacterial activity assays, all the fractions obtained from the dichloromethane sub-extract of *G. polycaulon* presented similar MIC values ($p=0.074$) (Table 1S). Concerning the ZI assay, the fractions **F1** (13.11-13.76 mm), **F3** (9.01-9.25 mm) and **F6** (8.42-8.91 mm) presented statistically significant higher activity than the other fractions and the positive control (Ofloxacin, 27.24-27.97 mm) ($p<0.001$) (Table 2S).

3.6. Cytotoxic activity of the fractions

All the fractions were significantly less cytotoxic than the positive control (ACT, $CC_{50}=0.01 \mu\text{g/mL}$) ($p<0.001$) (Table 9S). Only the fractions **F1** ($CC_{50}=86.17-86.92 \mu\text{g/mL}$), **F3** ($CC_{50}=82.91-83.12 \mu\text{g/mL}$) and **F6** ($CC_{50}=79.45-79.82 \mu\text{g/mL}$) were less cytotoxic than negative control (untreated cells) ($p<0.001$).

3.7. Anti-inflammatory activities of the fractions

As shown in Table 13S, the fractions obtained from the dichloromethane sub-extract have similar potential of inhibiting the NF- κ B production ($p=0.067$). The pharmacological assays (MIC, ZI, cytotoxicity and inhibition of NF- κ B production) and the chemical profile studies (^1H NMR spectra analysis) showed that fractions **F1**, **F3** and **F6** were the most active,

thus they were used for chromatographic separation obtaining compounds **1** (sub-fraction F1b), **2** (sub-fraction F3d) and **3** (sub-fraction F6c) (Tables 5S-16S).

3.8. Antibacterial activities of the compounds

Compounds **2** and **3** showed MIC values of 0.017-0.021 μ M and 0.0077-0.0079 μ M against *H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pneumoniae*. These values were statistically significantly higher than the positive control (Ofloxacin, MIC=27.64-27.67 μ M) ($p<0.001$). Compound **1** (MIC=44.80-44.85 μ M) had lower antibacterial activity than the positive control ($p<0.05$) (Figure 2).

Figure 2.

The ZI assay corroborated the antibacterial activity results. Compound **3** (11.36-11.67 mm) showed statistically significant higher inhibition potential than the positive control (Ofloxacin, 23.41-24.12 mm) ($p<0.001$) (Figure 3). However, compounds **1** (28.39-29.76 mm) and **2** (26.47-27.64 mm) had the same bacterial growth inhibition diameter (ZI) as the positive control (Ofloxacin, 23.41-24.12 mm) ($p=0.072$).

Figure 3.

3.9. Cytotoxic activity of the compounds

Table 5 includes the cytotoxicity values of the compounds, showing that compounds **2** (CC₅₀=47.04-47.47 μ M) and **3** (CC₅₀=32.48-34.63 μ M) have a statistically significant higher cytotoxicity than compound **1** (CC₅₀=92.79-94.91 μ M) ($p<0.001$). Moreover, compounds **2** and **3** are significantly less cytotoxic the positive control (ACT, CC₅₀=0.008 μ M) ($p<0.001$).

Table 5.

3.10. Anti-inflammatory activities of the compounds

The three compounds showed inhibitory capacity of the NF- κ B production, compounds **3** (IC_{50} =0.0071-0.0073 μ M) and **2** (IC_{50} =0.016-0.019 μ M) being the most promising, with concentrations of two (compound **3**) and three orders (compound **2**) of lower magnitude than the positive control (Celastrol, IC_{50} =7.41 μ M) (p <0.001) (Figure 4).

Figure 4.

4. Discussion

The antibacterial activity shown by the dichloromethane sub-extract at concentrations between 20.32-20.90 μ g/mL was higher than those reported by Kaminidevi et al. (2015) and Shanmugapriya et al. (2014; 2016) that studied the *n*-hexane extract from *G. polycaulon* against *A. fumigatus*, *A. oryzae*, *B. cereus*, *C. albicans*, *E. coli*, *Flavobacterium sp.*, *L. monocytogenes*, *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *Y. enterocolitica* showing ZI with values of 19-23 mm at a concentration of 50 μ g/mL. On the other hand, Shanmugapriya et al. (2014) reported that the methanolic extract from *G. polycaulon* presented antibacterial activity against the same bacteria in concentrations between 0.06-500 μ g/mL.

Thus, our research has shown that the dichloromethane sub-extract has more antibacterial potential than the *n*-hexane and methanol extracts of *G. Polycaulon*. This might be due to the type of compounds that can be extracted with organic solvents (Van Ngo et al., 2017). In our case, by analysing the proton spectrum of the dichloromethane sub-extract (Figure 3S), we can conclude that it is made up of aromatic compounds that contain nitrogen and sulphur thus

leading to a more promising antibacterial potential given the properties of these compounds (Radulović et al., 2013).

Regarding the antibacterial activity of the isolated compounds, there are no previous reports. In our study, compound **3** showed higher antibacterial activity ($IC_{50}=0.0077-0.0079 \mu M$), which may be due to the presence of its thiazole nucleus. This statement is based on the fact that there are reports of compounds with antibacterial activity that present this chemical nucleus (Jadhav et al., 2021). Finally, compound **1** showed lower activity due to the low capacity to cross membranes given its low lipophilicity (Miller et al., 2020).

The higher antibacterial capacity of the dichloromethane sub-extract (10.06-10.93 mm), observed in the ZI assay compared to that of compounds **1** (28.39-29.76 mm), **2** (26.47-27.64 mm) and **3** (11.36-11.67 mm) suggest that the presence of the three compounds in the sub-extract might generate a synergy between them, thus leading to a higher pharmacological potential than the one of the individual compounds.

The compounds isolated from *G. polycaulon* are azole derivatives that act at the level of the bacterial membrane. The site of action of these compounds can be on the hopanoids (diplopterol or amino-functionalised methylbacteriohopanepolyols which are the most abundant), which are lipids that act giving fluidity, symmetry and integrity to the bacterial membrane. In addition, they contribute to the proper functioning of many enzymes such as chitinsynthetase, which is important for the growth and division of the cell itself (Mangiarotti et al., 2019). This type of compounds (thiazole derivatives) inhibits the oxidative enzymes associated with the cytochrome P450 (CYP 3A4 and CYP 2C9), blocking the conversion of hopanoids into cholesterol, and leading to the alteration in the permeability of the bacterial cell membrane. In addition, they promote the accumulation of hydrogen peroxide capable of damaging the structure of the intracellular organelles of the bacteria (Shafiei et al., 2020).

There are no previous reports on the cytotoxicity of the *G. polycaulon* extracts. However, concerning the cytotoxicity of the compounds, previous studies have looked at compound **3**, which showed activity when tested in the Panc-1 (17.6 ng/mL) and BxPC-3 (30.5 ng/mL) cell lines while being highly cytotoxic (Zhang et al., 2015). In our case, compound **3** (CC₅₀=32.48-34.63 μ M) is indeed the most cytotoxic of the three compounds, but comparing with the positive control (ACT, 0.008 μ M), it is less cytotoxic. The concentrations differ from those reported by Zhang et al. (2018), and this difference may be due to the tested cell lines in the two studies (difference in morphological characteristics).

There are no previous pharmacological studies on the anti-inflammatory activity of the aqueous extract of *G. polycaulon*. However, other species of the genus *Gnaphalium* showed anti-inflammatory activity, as reported by Zheng et al. (2013) who indicated that the methanolic extract of *G. stramineum* had high anti-inflammatory potential.

There are no reports on the anti-inflammatory potential of the isolated compounds with the only exception of Lowin et al. (2015) that reported no anti-inflammatory effect of compound **2** on the secretion of interleukin-6 (IL-6), interleukin-8 (IL-8) and matrix metalloproteinase-3 (MMP-3). In our study, compound **2** had a high anti-inflammatory activity (IC₅₀=0.016-0.019 μ M), inhibiting the NF- κ B production, which is related to the IL-6, IL-8 and MMP-3 secretion (Brasier, 2010).

Most microorganisms (bacteria) activate the NF- κ B pathway through molecular pattern receptors associated with patterns called Toll-like receptors. There are different compounds that can affect different parts of the NF- κ B pathway, including DNA binding, nuclear translocation, and I κ B α phosphorylation/degradation (Gilmore & Herscovitch, 2006). In the case of compounds derived from thiazoles, Rokde et al. (2020) and Petrou et al. (2021) indicated that this type of chemical structures has anti-inflammatory activity. The possible site

of action of these compounds may be in the DNA binding domain of NF- κ B interacting with Tyr57, Val58, Cys59, His141 and Val142 (Singh et al., 2017).

Likewise, a relationship was observed between the pharmacological activity and the lipophilicity of the compounds (Miller et al., 2020). This is because the pharmacological potential of compounds is determined by their ability to be transported through lipid structures, such as the cell membrane (Apaza et al., 2021). In this sense, the logP is a physicochemical parameter that allows to quantitatively determine the degree of lipophilicity of a compound. In our research, compound **1** (logP=-0.72) was less active, due to its low logP, compared to compounds **2** (logP=3.13) and **3** (logP=3.95) which have higher lipophilicity and higher logP, thus confirming the importance of the link between activity and lipophilicity.

Limitations of the current study include the fact that it was not possible to perform antibacterial and anti-inflammatory activity assays of the three combined compounds to determine the synergism that exists between them. This hypothesis arises from the observation that the dichloromethane sub-extract showed higher values of antibacterial and anti-inflammatory activity than the fractions, and as the sub-extract was fractionated, the activities decreased, indicating that there is a synergism between the compounds of the dichloromethane sub-extract.

4. Conclusion

In this research, the antibacterial and anti-inflammatory activities of the aqueous extract of *Gnaphalium polycaulon* have been showcased, thus confirming the traditional use of this plant. In addition, it is the first time that 2-(4-(1-H-tetrazol-1-yl) phenyl)-2-aminopropanoic acid (**1**), *N*-phenyl-4-(3-phenyl-1,2,4-thiadiazol-5-yl) piperazine-1-carboxamide (**2**) and *N*-(4-ethoxyphenyl)-4-(2-methylimidazo-[1,2- α] pyridine-3-yl) thiazol-2-amine (**3**) have been isolated from *Gnaphalium polycaulon*.

This means that both the aqueous extract and the compounds could be used against infections in the respiratory tract to inhibit the pathogenic agent (bacteria) and treat inflammation in the infected area, being an alternative to the antibiotics that already exist on the market.

Finally, it was determined that the lipophilic capacity plays an important role in the antibacterial and anti-inflammatory effect of the compounds. In this sense, if we are to carry out chemical modifications on the acid group of the compound **1**, such as an esterification or amidation, the lipophilicity might increase, leading to a higher activity potential.

Declaration of interest

The authors declare no conflict of interest.

Author contributions

H.P.B and A.T.L performed the phytochemical analysis, isolation and characterisation of the compounds. P.M.M.J and O.D.M performed the biological experiments. P.M.M.J assisted with the writing of the manuscript and the preparation of the figures. R.S.A conceived and supervised the study, provided the materials and reagents. A.T.L. conceived and supervised the study, performed the experiments and statistical analysis, and wrote and edited the manuscript and figures. All authors read and approved the final manuscript.

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Supplementary Material

¹H- and ¹³C- NMR, ¹H-¹H COSY, HSQC, HMBC and MS spectra for the different fractions assayed in this study, together with isolated compounds **1-3** are provided as supporting information (Figures 5S-36S). The activity data of the *G. polycaulon* fractions from the different samples are provided as supporting information (Tables 1S-16S).

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Figures captions

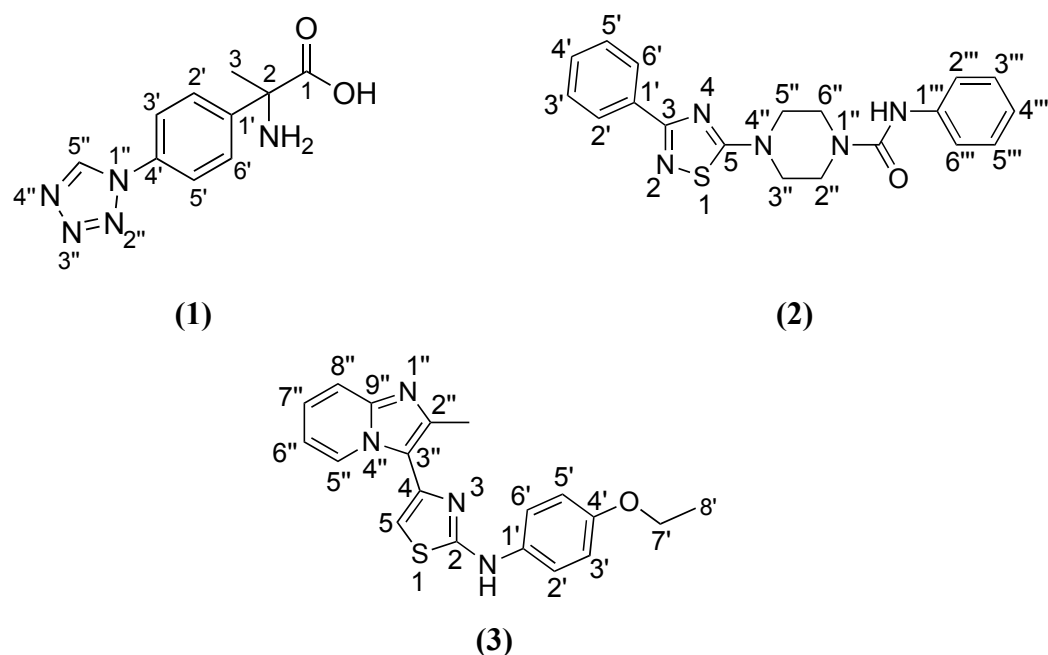


Figure 1. 2-(4-(1H-tetrazol-1-yl) phenyl)-2-aminopropanoic acid (1), *N*-phenyl-4-(3-phenyl-1,2,4-thiadiazol-5-yl) piperazine-1-carboxamide (2) and *N*-(4-ethoxyphenyl)-4-(2-methylimidazo-[1,2- α] pyridine-3-yl) thiazol-2-amine (3).

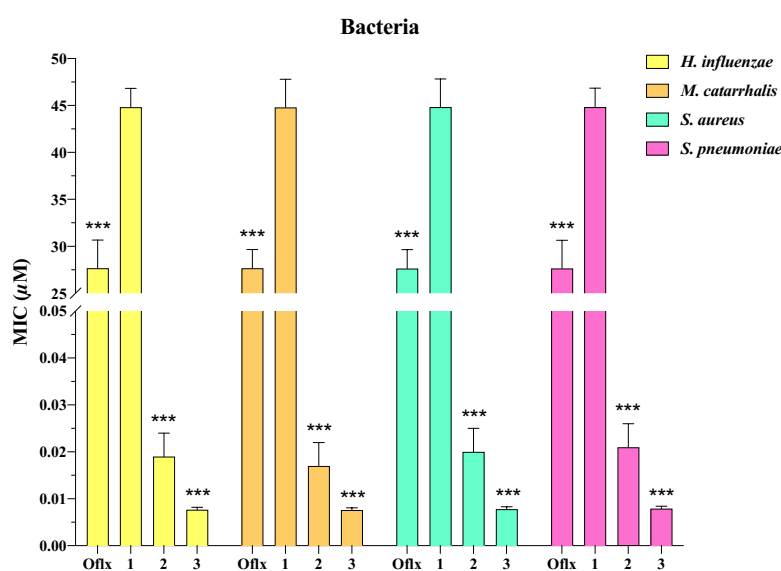


Figure 2. Minimum inhibitory concentration (MIC) average values of *G. polycaulon* compounds against different strains of bacteria at 48 h. **Oflox**=Ofloxacin (positive control); **1**=Compound 1; **2**=Compound 2; **3**=Compound 3. Confidence interval 95%/Tukey's multiple comparisons test (***) $p < 0.001$.

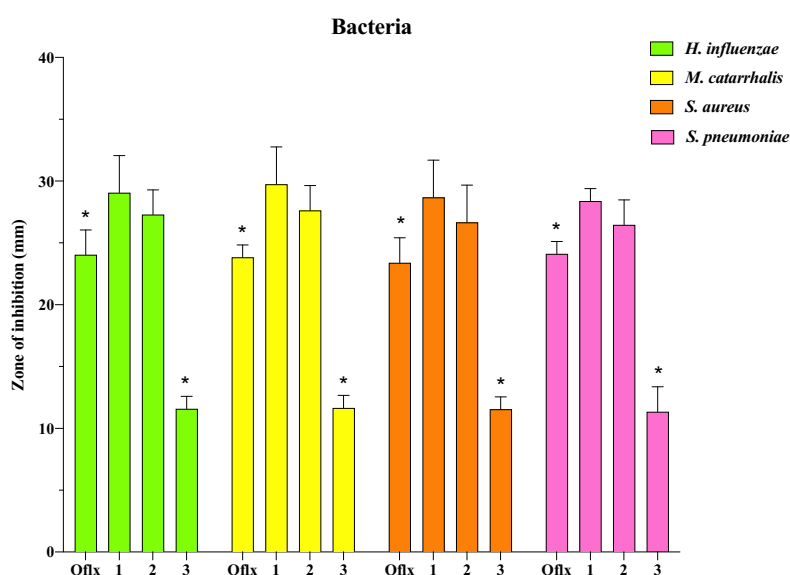


Figure 3. *In vitro* culture plates (agar cup plate method) of *G. polycaulon* compounds showing the average values of the zone of inhibition (ZI) concentration against different strains of bacteria at 48 h. **Oflox**=Ofloxacin (positive control); **1**=Compound 1; **2**=Compound 2; **3**=Compound 3. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

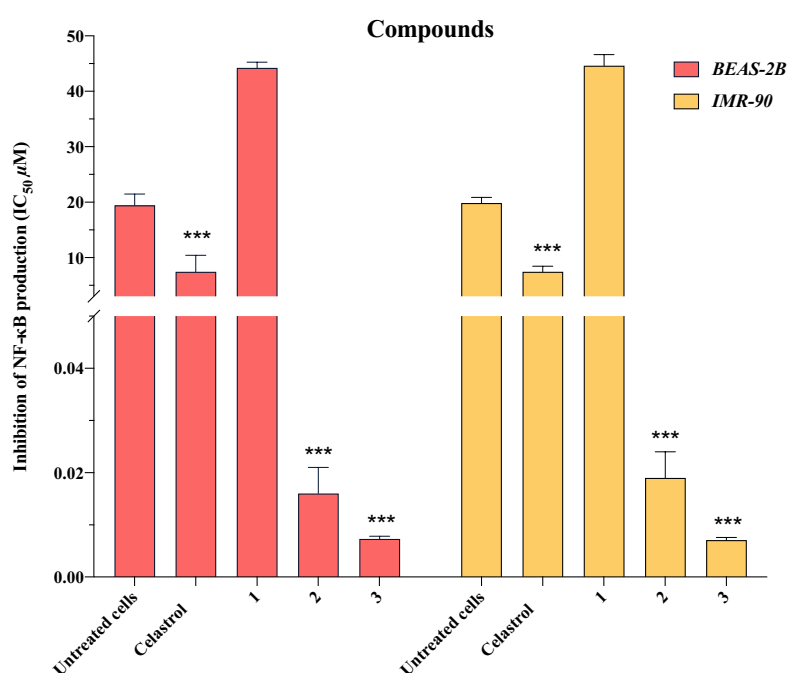


Figure 4. IC₅₀s average values of the inhibition of NF-κB production, calculated for the compounds from *G. polycaulon* at 48 h. **Untreated cells**=Negative control, **Celastrol**=Positive control; **1**=Compound 1; **2**=Compound 2; **3**=Compound 3. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

Tables captions

Table 1. Minimum inhibitory concentration (MIC) average values of *G. polycaulon* extracts against different strains of bacteria. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

Extracts	Minimum inhibitory concentration at (MIC $\mu\text{g/mL}$) at 48 h			
	<i>H. influenzae</i>	<i>M. catarrhalis</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>
Ofloxacin	10.01 \pm 1.87	10.01 \pm 1.86	10.01 \pm 1.85	10.01 \pm 1.83
Aq extract	40.32 \pm 3.47	40.24 \pm 3.48	40.84 \pm 3.50	40.95 \pm 3.45
HEX sub-extract	80.52 \pm 4.12	80.16 \pm 4.05	80.75 \pm 4.37	80.79 \pm 4.39
DCM sub-extract	20.58 \pm 2.67	20.32 \pm 2.33	20.58 \pm 2.69	20.90 \pm 2.61
MeOH sub-extract	75.05 \pm 4.01	75.49 \pm 4.09	75.70 \pm 4.16	75.78 \pm 4.15

Table 2. *In vitro* culture plates (agar cup plate method) of *G. polycaulon* extracts showing the average values of the zone of inhibition (ZI) concentration against different strains of bacteria. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

Extracts	Zone of inhibition at (mm) at 48 h			
	<i>H. influenzae</i>	<i>M. catarrhalis</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>
Ofloxacin	27.77 \pm 3.03	27.89 \pm 3.02	27.13 \pm 2.58	27.12 \pm 2.50
Aq extract	41.67 \pm 3.36	41.82 \pm 3.44	41.20 \pm 3.15	41.13 \pm 3.17
HEX sub-extract	93.61 \pm 4.35	93.94 \pm 4.49	93.47 \pm 4.14	93.24 \pm 4.17
DCM sub-extract	10.93 \pm 1.12	10.94 \pm 1.16	10.36 \pm 1.05	10.06 \pm 1.03
MeOH sub-extract	70.69 \pm 2.25	70.71 \pm 2.26	70.46 \pm 2.14	70.35 \pm 2.12

Table 3. CC₅₀s average values of the LDH (Cytotoxicity) assays calculated for the extracts from *G. polycaulon*. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

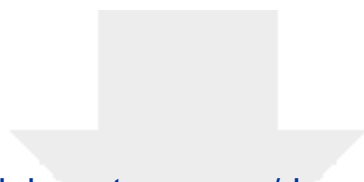
Extracts	Cytotoxicity (CC ₅₀ $\mu\text{g/mL}$) at 48 h	
	BEAS-2B	IMR-90
Untreated cells	98.47 \pm 4.74	97.05 \pm 4.73
DMSO	20.15 \pm 2.39	20.12 \pm 2.33
Actinomycin D	0.01 \pm 0.008	0.01 \pm 0.008
Aq extract	93.68 \pm 4.14	92.88 \pm 4.38
HEX sub-extract	76.24 \pm 3.75	76.13 \pm 3.73
DCM sub-extract	86.98 \pm 3.91	86.49 \pm 3.62
MeOH sub-extract	68.83 \pm 2.41	68.39 \pm 2.15

Table 4. IC₅₀s average values of the inhibition of NF- κ B production, calculated for the extracts from *G. polycaulon*. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (** $p < 0.001$).

Extracts	Inhibition of NF- κ B production (IC ₅₀ $\mu\text{g/mL}$) at 48 h	
	BEAS-2B	IMR-90
Untreated cells	19.98 \pm 1.23	19.94 \pm 1.26
Celastrol	3.34 \pm 0.08	3.32 \pm 0.05
Aq extract	42.92 \pm 3.43	42.77 \pm 3.34
HEX sub-extract	83.72 \pm 3.57	82.89 \pm 3.56
DCM sub-extract	21.71 \pm 1.32	20.03 \pm 1.18
MeOH sub-extract	77.52 \pm 3.34	76.62 \pm 3.27

Table 5. CC₅₀s average values of the LDH (cytotoxicity) assays calculated for the compounds from *G. polycaulon*. CI95%: Confidence interval 95%/Tukey's multiple comparisons test (***p*<0.001).

Compounds	Cytotoxicity (CC ₅₀ μ M) at 48 h	
	BEAS-2B	IMR-90
Untreated cells	99.93 \pm 3.46	98.83 \pm 3.49
DMSO	10.05 \pm 1.26	10.05 \pm 1.24
Actinomycin D	0.008 \pm 0.0005	0.008 \pm 0.0005
Compound 1	92.79 \pm 3.96	94.91 \pm 3.94
Compound 2	47.04 \pm 3.13	47.47 \pm 3.75
Compound 3	34.63 \pm 2.49	32.48 \pm 2.36



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Supplementary Material

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