

In vitro Antibacterial, Antioxidant and Anti-Inflammatory Effects of *Senecio asperulus* and *Gunnera perpensa* from Mohale's Hoek, Lesotho

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ABSTRACT

Background: Traditional medicinal plants have been widely used to treat or manage various ailments for centuries in Lesotho. With an increase in multi drug resistance and undesired adverse events to current drugs challenges, there is a need for alternative drugs. **Aim:** In this study we aimed at the investigation of antibacterial, antioxidant and anti-inflammatory effects of *Senecio asperulus* and *Gunnera perpensa* roots extracted in three solvents of different polarities. **Materials and Methods:** Antibacterial activity was determined using the disc diffusion method, while antioxidant activity was determined using free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl and the ferric reducing antioxidant power assay. The Lipopolysaccharide (LPS) stimulated RAW 264.7 mouse macrophage *in vitro* model was used to evaluate the anti-inflammatory activity of both plants. Resveratrol was used as a positive control. **Results:** Methanol extracts of *Senecio asperulus* inhibited microbial growth even at the lowest concentration of 50 µg/ml. *Senecio asperulus* dichloromethane extract was active on most bacteria with MIC's between 50 µg/ml and 500 µg/ml. However, the water and methanol extracts of *Gunnera perpensa* had no activity against all organisms tested. Aqueous extracts of *Senecio asperulus* and *Gunnera perpensa* showed free radical scavenging activity yielding EC₅₀ values of 100 µg/ml and 25 µg/ml, respectively. The aqueous extracts of *Senecio asperulus* showed moderate anti-inflammatory activity from 50 to 200 µg/ml. while the methanol extract was at 200 µg/ml and with no cytotoxicity. No anti-inflammatory activity was observed from all *Gunnera perpensa* extracts using LPS-induced macrophages, this suggests that this species may be using other mechanisms for anti-inflammatory activity. **Conclusion:** The antibacterial, antioxidant and anti-inflammatory activities observed from water extracts of *Senecio asperulus* support its ethnomedicinal use for the management of inflammation related diseases.

Key words: Antibacterial activity, Antioxidant activity, Anti-inflammatory activity, *Senecio asperulus*, *Gunnera perpensa*, Medicinal plants.

key messages: In Mohale's Hoek, Lesotho, *Senecio asperulus* and *Gunnera perpensa* are used for the treatment or management of various ailments especially since access to western health care facilities is limited. We are reporting on the efficacy of *Senecio asperulus* aqueous and methanol extracts as good candidates for potent novel anti-inflammatory, multi-drug resistant bacteria and antioxidant drugs development, as they had no cytotoxicity effects on tested RAW 264.7 cells. It is important to validate the ethnopharmacology and pharmacognosy of crude extract instead of isolated compounds as most active ingredients from medicinal plants work in synergy, hence crude extracts were used in this study.

INTRODUCTION

Inflammation is the protective response to injury of body cells and tissues due to exposure to various factors like infections, chemicals, heat and mechanical injuries. Chronic inflammation could be considered the basis of disease and this is a typical feature of many chronic disorders. Infections by organisms which are resistant to killing and clearing by the body, tend to cause chronic inflammation. Cell walls of gram-negative bacteria have lipopolysaccharides (LPS), which are endotoxins that have the ability to cause inflammation. The host response to LPS is known to be mediated by reactive oxygen species (ROS) as they lead to Rac activation and IL-1 expression, which are required

to induce inflammation response.¹⁻³ Excessive reactive oxygen species are closely involved in various human diseases such as inflammation, cancer, heart disease, aging, atherosclerosis, rheumatoid arthritis and Alzheimer's disease. Antioxidants have an ability to neutralize excessive ROS which can induce the oxidative stress that causes cell damage and culminates in inflammation.

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent/chronic inflammation ultimately resulting in tissue dysfunction. The inflammation process triggers the release of mediators; histamine, kinins and prostaglandins, by damaged tissues which in

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turn involves cell membrane alterations, vascular permeability and increased protein denaturation.⁴ Inflammation mediators are released as a result of induced cyclooxygenase 2 (COX-2), nitric oxide (NOS) and lipoxygenase (LP). Most non-steroidal anti-inflammatory drugs inhibit pathways that lead to the generation of inflammation mediators and this is essential for the treatment of inflammation. A good non-steroidal anti-inflammatory drug (NSAIDs) acts through the inhibition of induced nitric oxide synthase (iNOS), which is a key enzyme for the generation of nitric oxide (NO) and cyclooxygenase (COX) isozymes. There is dissatisfaction with existing anti-inflammatory drugs, as they impose toxicity and adverse side effects, particularly affecting the gastrointestinal and cardiovascular systems.⁵ Moreover, they lead to much dependence on them, as disorders reoccur after treatment has been discontinued. The bad side effects from the synthetic anti-inflammation drugs that are currently available have triggered a global trend of returning to natural sources of medicines. Thus, there is a significant increase in scientific and commercial interest in the discovery of new anti-inflammatory therapeutic and preventative agents from natural product sources.

Medicinal plants play a vital role in Lesotho's primary healthcare system as they are components of the most diverse and oldest African therapeutic systems. People in Lesotho use medicinal plants for self-medication and they can diagnose and treat minor ailments without even consulting a traditional doctor or herbalist.⁶ This occurs particularly in remote rural areas with limited access to health facilities. Local people in these areas rely on a variety of medicinal plants such as *Senecio asperulus* and *Gunnera perpensa* to manage different ailments including inflammation related diseases.^{7,8} *Senecio asperulus* is a member of the Asteraceae family and is known as *Moferefere* or *Letapisa* by the Basotho people in Lesotho. *Gunnera perpensa* known as *Qobo* belongs to the Gunneraceae family. The dried roots of both plants are extensively used, independently or as a mixture with other medicinal plants, to treat a variety of ailments. Their uses include, but not limited to; colds and flu, urinary tract infections, sexual transmitted diseases, reproductive healthcare, umbilical cord inflammation, as vasodilating agents and circulation improvers.^{8,9} However, there is limited scientific data to validate these ethnobotanical claims is scarce. Thus, this study was carried out to validate and justify the traditional claims for the use of *Qobo* and *Moferefere* in the treatment of inflammation related diseases.

METHODS AND MATERIALS

Plant collection

The study received a plant collection and export permit from the Ministry of Tourism Environment and culture, Lesotho and import approval (NC.553/2017) from The Department of Economic Development, Tourism and Environmental Affairs, South Africa. Plant material was collected from the mountains of Lesotho (Mohale's Hoek district) and authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa where voucher specimens, PHM01 for *Senecio asperulus* and PHM02 for *Gunnera perpensa* were deposited.

Preparation of plant extracts

The roots of both plant species were washed, air dried at room temperature and then ground into fine powder using an electric blender. The powdered roots of *Senecio asperulus* and *Gunnera perpensa* were soaked separately with distilled water (DH₂O), methanol (MeOH) and dichloromethane (DCM) for 72 h with occasional stirring. After filtration, the aqueous extracts were freeze dried and the organic solvent extracts were concentrated with rocket evaporator. Thereafter, the extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL respectively. Stock solutions were

sonicated were solubility was an issue. All samples were then stored at 4°C.

Cell culture

The mouse macrophage cell line, RAW 264.7 were cultured and maintained in RAW 264.7 growth medium 1 (RAWGM1) at 37°C in a humidified atmosphere with 5% CO₂. Suspensions of RAW 264.7 monolayer culture were seeded into 96 well microtiter plates at a density of 25,000 cells per well using a volume of 50 µl in each well. The microtiter plates were then incubated at 37°C, 5% CO₂ and 100% relative humidity for 24 h prior to addition of test compounds to allow for cell attachment. The culture medium was then removed and the samples were added to give final concentrations of 12.5 and 50 µM.

Microorganisms

The American Type Culture Collection (ATCC) microorganisms used in this study were obtained from the Pathcare microbiology laboratory in Welkom South Africa. Four Gram positive bacteria (*Staphylococcus aureus* ATCC BAA-1026, *Staphylococcus saprophyticus* ATCC BAA-750, *Enterococcus faecalis* ATCC 29212 and *Streptococcus pneumoniae* ATCC 49619) and four Gram negative bacteria (*Enterobacter cloacae* ATCC 700323, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 35659 and *Klebsiella pneumoniae* ATCC 700603) were inoculated onto Muller-Hinton (MH) agar and later used in this study. All bacterial suspensions were prepared using sterile saline to obtain an optical density comparable to the density of 0.5 McFarland barium sulphate standard (turbidity = 108 CFU/mL).

Phytochemical screening assay

The qualitative phytochemical screening for the presence of flavonoids, tannins, saponins, terpenoids and alkaloids was carried out following methods by Shanmugam *et al.*¹⁰

In vitro antibacterial assay

The antibacterial activity was conducted using the disc diffusion method as adopted from Thitilertdecha *et al.*¹¹ and Su *et al.*¹² To determine the minimum inhibition concentration (MIC) of each plant extract, selected Gram positive and Gram-negative micro-organisms were treated with 4 concentrations of plant extracts from serial dilution namely: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. After serial dilutions were prepared, 6 mm filter paper discs were impregnated with the extract at each concentration. The extract-loaded discs were then placed on the surface of the agar inoculated with different micro-organisms as mentioned previously. Plates were incubated aerobically at 37°C and zones of inhibition were measured after 24 h. The zones of inhibition were measured in mm using a calliper and the lowest concentration at which growth inhibition resulted was considered as minimum inhibition concentration (MIC). Discs (6 mm filter paper discs) dipped in DMSO were used as negative controls, while commercial chloramphenicol discs (25 µg) and ampicillin (10 µg), were used as positive controls. The tests were performed in triplicates and mean was calculated and reported.

In vitro anti-oxidant assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was performed using a method by Najafabad and Jamei.¹³ Two hundred micro-litres (200 µL) of 0.1 mM DPPH prepared in methanol was added to 100 µL of the plant extract diluted in different concentrations. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was then measured at 517 nm. Ascorbic acid was used as a positive control. The experiments were performed in triplicates and percentage inhibition of the DPPH radical scavenging activity was calculated using the formula:

$$\% \text{ Inhibition} = ((Ac - A_1) / Ac) \times 100$$

Where Ac is the absorbance of the control and A₁ is the absorbance of the sample.

Estimation of reducing power

The reducing power of the plant extracts was estimated using the method by Najafabad and Jamei,¹³ whereby 500 µl of 0.2 M phosphate buffer (pH-6.6), 500 µl of ferricyanide (1% w/v) were mixed with 200 µl of each extract and the mixture was then incubated for 20 min at 50°C. After incubation, 500 µl of TCA (10% w/v) was added to the extract mixture and centrifuged for 10 min at 3000 rpm. Thereafter, 500 µl of the supernatant was collected using an automated pipette and added to 500 µl of distilled water together with 100 µl of ferric chloride (0.1% w/v) and mixed thoroughly. Absorbance was then measured at 700 nm wavelength. Percentage inhibition was calculated for the determination of reducing power:

$$\% \text{ Inhibition} = (Ac - A_1) / Ac \times 100$$

Where A₁ is the absorbance of the sample, Ac is the absorbance of control.

Determination of anti-inflammatory activity in RAW 264.7 macrophages

To assess the anti-inflammatory activity of the plant extracts, 50 µl of Lipopolysaccharide (LPS) containing medium was added to the 96-wells plates with RAW 264.7 treated with plant extracts (as mentioned above under cell culture). Cells were then incubated for 20 h. To quantify nitric oxide (NO) production, 50 µl of the above culture medium with LPS was transferred to a new 96-well plate and 50 µl Griess reagent added. Absorbance was then measured at 540 nm wavelength and the results were expressed relative to the appropriate untreated control. A well-known inhibitor of Inducible nitric oxide synthase (iNOS) expression, resveratrol, was used as a positive control.

MTT assay

To confirm that toxicity was not a contributory factor for anti-inflammatory activity, cell viability was assessed using the MTT (3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay.¹⁴ RAW 264.7 cells were seeded in a 96 well microtiter plates at a density of 25,000 cells per well using a volume of 50 µl in each well and allowed to adhere for 24 h at 37°C in a CO₂ incubator. Media was then carefully aspirated from adherent cell cultures, discarded and replaced with fresh media. Cells were then treated with various concentrations of the plant extracts and re-incubated for 24 h at 37°C. After incubation, the culture media was aspirated again and replaced with equal volumes of fresh media and MTT working solution (5 mg/mL in phosphate buffer solution). Plates were then incubated further for 4 h at 37°C. The media with MTT was removed and 100 µl of DMSO solution was added into each well to solubilize the formed MTT formazan crystals (purple colour). Absorbance was measured at 570 nm wavelength and cell inhibition percentage was determined using the formula:

$$\text{Percentage Cell Inhibition} = [100 - \text{Abs (sample)} / \text{Abs (control)}] \times 100.$$

Statistical analysis

All assays were performed in triplicate in all independent and separate experiments. The data is presented as means ± SD from three independent analyses and separate experiments. One-way analysis of variance (ANOVA) was applied to determine the statistical significance in various anti-inflammatory and cytotoxicity markers level between the control and the tested samples. Microsoft EXCEL software was used. The level of significance will be set at 0.05 and 0.01.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemicals are naturally produced by plants for their protection and as a defence mechanism against microbes and insects. These phytochemicals can either be primary compounds such as, chlorophyll, proteins and common sugars, or be secondary compounds such as terpenoids, alkaloids, flavonoids, reducing sugars, tannins and phenols.¹⁵ Secondary metabolites are known to have important medicinal benefits to humans.¹⁶ Moreover, many studies have proved the diverse pharmacological activities of terpenoids as anti-bacterial, anti-oxidant, anti-inflammatory, anticancer, anti-viral, anti-malarial and anaesthetic agents.¹⁷⁻²⁰ Geographical properties, natural conditions and bioconstituents of soil, does influence quality of secondary metabolites.^{21,22} This is indeed true, as *Senecio asperulus* collected from Mohale's Hoek, Lesotho (more rural area) was found to be rich in secondary metabolites when compared with phytochemistry findings by Mugomeri *et al.*⁷ using the same plant but collected from Maseru, Lesotho (which is the capital city of the Basotho country). However, our findings corroborated with Mugomeri *et al.*⁷ findings as *Senecio asperulus* methanol extracts had flavonoids, of which the aqueous extracts of the same plant showed no presence of flavonoids. This simply means that *Senecio asperulus* methanolic extracts contain flavonoids, regardless of their point of collection. From our results, terpenoids were present from all extracts of both plant species, extracted in different solvents, thus supporting the traditional medicinal values of these plants (Table 1). Aqueous extracts of *Senecio asperulus* showed the presence of more phytochemicals when compared to methanolic and dichloromethane extracts of the same plant species. *Gunnera perpensa* water extracts showed only the presence of flavonoids, terpenoids and tannins. However, its methanolic and dichloromethane extracts revealed the presence of almost all investigated phytochemicals (Table 1). This discovery indicates that active ingredients from this plant are more hydrophobic thus extracted better with solvents which are non-polar.

Antibacterial activity

To determine the antibacterial activity and minimum inhibitory concentration (MIC) of each plant extract, selected Gram-positive and Gram-negative micro-organisms were treated with different concentrations of plant extracts. The zone of inhibition was measured and compared to that of controls. Two known antibiotics namely; ampicillin (for the Gram-positive organisms) and chloramphenicol (for the Gram-negatives) were used as positive controls, while saline was used as a negative control. MIC of each active extracts were presented in Table 2. Antibacterial bioactive plant compounds affect the integrity of the bacterial cell wall permeability so the bacteria leak to death, thus, inhibiting bacterial growth.²³ The methanolic extracts of both *Senecio asperulus* and *Gunnera perpensa* failed to inhibit the growth of the Gram positive and negative microorganisms selected for this study even at higher concentrations of 500 µg/ml, except for *S. faecalis* (Table 2). The most active plant extract was *Senecio asperulus* aqueous extract as it managed to inhibit growth of all strains tested against. *Gunnera perpensa* dichloromethane extracts also inhibited most microorganisms and only one organism (*S. faecalis*) was resistant even at the highest concentration. The antibacterial activity of (*Senecio asperulus* aqueous extract and *Gunnera perpensa* dichloromethane extract) relates bioactivity to the phytochemical content (Tables 1 and 2).

Minimum inhibition concentration (MIC) required to inhibit *S. faecalis* was 500 µg/ml for *Senecio asperulus* methanol extract and 50 µg/ml for the aqueous and DCM extracts. Aqueous extracts further showed MIC to be 50 µg/ml for most tested Gram-negative's and Gram-positive's, except for *S. aureus* and *S. saprophyticus*, as their MIC 's were at 100 µg/ml and 250 µg/ml, respectively (Table 2). *Senecio asperulus*

Table 1: Phytochemical screening analysis of *Senecio asperulus* and *Gunnera perpensa*. (+) Indicate the presence and degree (due to color intensity) of phytochemicals and (-) Indicate the absence of phytochemicals.

Plant extract	Flavonoids	Tannins	Saponins	Terpenoids	Steroids	Alkaloids
<i>Senecio asperulus</i> (MeOH)	++	++	-	+	+	-
<i>Senecio asperulus</i> (H ₂ O)	-	+	+	+	++	+
<i>Senecio asperulus</i> (DCM)	+	-	-	++	+	+
<i>Gunnera perpensa</i> (MeOH)	+	+	+	++	-	+
<i>Gunnera perpensa</i> (H ₂ O)	+++	+	-	+	-	-
<i>Gunnera perpensa</i> (DCM)	+++	-	+	++	-	++

Table 2: Antibacterial analysis of *Senecio asperulus* and *Gunnera perpensa*.

Micro-organisms	Concentration (µg/ml)	S1	S2	S3	G1	G2	G3	Control A	Control B
<i>S. faecalis</i>	500	+	++	+	-	-	-		
	250	-	+	+	-	-	-	++	-
	100	-	+	+	-	-	-		
	50	-	+	+	-	-	-		
<i>S.pneumoniae</i>	500	-	+	-	-	-	+		
	250	-	+	-	-	-	+	++	-
	100	-	+	-	-	-	+		
	50	-	+	-	-	-	+		
<i>P.mirabilis</i>	500	-	++	+	-	-	++		
	250	-	+	+	-	-	+		
	100	-	+	+	-	-	+	-	++
	50	-	+	+	-	-	-		
<i>K.pneumoniae</i>	500	-	++	+	-	-	++		
	250	-	+	+	-	-	++		
	100	-	+	+	-	-	+	-	++
	50	-	+	+	-	-	+		
<i>S.saprophyticus</i>	500	-	+	-	-	-	+		
	250	-	+	-	-	-	+	++	-
	100	-	-	-	-	-	+		
	50	-	-	-	-	-	+		
<i>S.aureus</i>	500	-	+	-	-	-	+		
	250	-	+	-	-	-	+	++	-
	100	-	+	-	-	-	-		
	50	-	-	-	-	-	-		
<i>E. coli</i>	500	-	+	+	-	-	+		
	250	-	+	+	-	-	+		
	100	-	+	+	-	-	+	-	++
	50	-	+	-	-	-	+		
<i>E.cloacae</i>	500	-	+	+	-	-	++		
	250	-	+	-	-	-	+		
	100	-	+	-	-	-	+	-	++
	50	-	+	-	-	-	+		

Abbreviations: S1: *Senecio asperulus* methanol extract; S2: *Senecio asperulus* aqueous extract; S3: *Senecio asperulus* dichloromethane extract; G1: *Gunnera perpensa* methanol extract; G2: *Gunnera perpensa* aqueous extract; G3: *Gunnera perpensa* dichloromethane extract; [++]: sensitive with zone of inhibition ≥ 16 mm; [+]: sensitive with zone of inhibition ≤ 16 mm; [-]: resistant with no zone of inhibition. **Control A:** Ampicillin; **Control B:** Chloramphenicol. MIC of each active extracts are presented with "Bold" cross signs (+) and yellow colouring.

further showed the MIC to be at 50 µg/ml for *S. faecalis*, *P. mirabilis* and *K. pneumoniae* and at 100 µg/ml for *E. coli*. *E. cloacae* resisted the DCM extract on all other concentrations, besides the highest tested concentration of 500 µg/ml, however, the zone of inhibition was > 16 mm. *Gunnera perpensa* DCM extract was very active with the MIC of 50 µg/ml for most organisms, besides *P. mirabilis* and *S. aureus* as their MIC's were 100 µg/ml and 250 µg/ml, respectively (Table 2).

Anti-oxidant activity

Biological oxidant reactions of exogenous and endogenous factors generate active oxygen species or oxidants such as free radicals in a human body. Unfortunately, these unstable produced oxidants give rise to oxidative stress and are found to be responsible for many diseases due to their ability to cause DNA damage. Phenols have redox properties which acts as hydrogen donors, oxygen quenchers and reducing agents, thus, delocalize the unstable and unpaired electron within the aromatic structure.²⁴ Antioxidants from natural sources are important in safeguarding human health and as protection from diseases resulting from oxidative stress. Phytochemical antioxidants such as flavonoids have an aptitude to reduce oxidative stress by neutralizing these reactive oxygen species. Moreover, natural antioxidants are considered to be more active and safer to health than synthetic antioxidants, as the latter turns to have detrimental side effects when used *in vivo*.¹⁷

The DPPH assay was used to investigate the free radical-scavenging activities of *Senecio asperulus* and *Gunnera perpensa*. Activities were expressed as percentages in order to determine plant extract concentration required to achieve a 50% DPPH scavenging activity (EC₅₀). The dose-dependent antioxidant activity of the tested extracts are summarized in Figure 1. The aqueous extract of *Senecio asperulus* had an EC₅₀ of 100 µg/ml and managed to scavenge about 78% DPPH at the highest concentration of 500 µg/ml. *Gunnera perpensa* aqueous extract EC₅₀ was observed at the lowest concentration of 25 µg/ml, whereas ascorbic acid EC₅₀ was only at 50 µg/ml, which is two-folds. This was very impressive as it suggests *Gunnera perpensa* to be a very strong antioxidant agent.

The genus *Senecio* is known to be rich in flavonoids²⁵ and *Gunnera perpensa* is the only species from the *Gunnera* genus which has flavonoids.²⁶ Flavonoids are known to have very strong antioxidant activities.^{27,28} Tannins are known as water-soluble phenolic compounds and were present in both plant species aqueous extracts. According to Karamac' *et al.*²⁹ tannins can chelate metal ions and interfere with the formation of hydroxyl free radicals, thus, cancels oxidation. There was a directly proportional relationship between the presence of both flavonoids and tannins and the antioxidant activity of *Senecio asperulus* methanol and *Gunnera perpensa* aqueous extracts. Thus, indicating that these phenols; flavonoids and tannins, were the leading contributors to the observed antioxidant activities.

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity as it proves the electron transfer ability of the extract. The reduction of oxidized intermediates of lipid peroxidation process was indicated by the formation of ferrous chloride and potassium ferrocyanide which produced a green to blue colour. The intensity of the formed colour was dependent on the reducing power of the extract used. *Senecio asperulus* and *Gunnera perpensa* aqueous and methanol extracts showed increased ferric reducing power with the increasing concentration as shown in Figure 2. The aqueous extracts from *Gunnera perpensa* proved to be a better source of antioxidants as their reducing power were 50% at 100 µg/ml, followed by the methanolic extracts as these extracts also showed increased reducing power. The dichloromethane extracts of both plants showed less reducing power than ascorbic acid and the other extracts. These results did indicate that *Senecio asperulus* and *Gunnera perpensa* have very strong free radical scavenging and antioxidant properties

(Figure 1) possibly due to their hydrogen donor ability as confirmed by the estimation of ferric reducing power.

Anti-inflammatory activity

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues including adipose. Moreover, they diversely perform complex microbicidal functions, such as; surveillance, chemotaxis, phagocytosis and destruction of pathogenic micro-organisms. Therefore, the development of therapeutics which can non-specifically enhance the innate immune response represents a promising strategy to combat classical and emerging infectious agents.³⁰ However, depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative. Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as lipopolysaccharides (LPS) and interferon gamma (IF-γ). These classically activated macrophages produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells.

In addition, these activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS).³¹ The mouse macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of test samples. As cells were cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production were determined by measuring the levels of nitrate in the culture medium. Figure 3 illustrates the levels of nitrate macrophage cells produce nitrite and nitrate in response to LPS formed after macrophages were exposed to plant extracts.

High anti-inflammatory activity was observed from the DCM extract of *Senecio asperulus*, even when compared with the activity of the control. *Senecio asperulus* showed an ability to suppress NO production by down-regulating the expression of iNOS in RAW 264.7 macrophages. According to Chen *et al.*³² non-lectin glycoprotein (PCP-3A) inhibits LPS-induced production of NO through the down-regulation of iNOS via a transcription factor NF-κB mechanism. Thus, this could suggest that anti-inflammatory activity observed from *Senecio asperulus* (Figure 3) may be through this mechanism involving the transcription factor NF-κB, however, this remains to be elucidated through further investigations. Furthermore, it is known that LPS induces the expression of iNOS through redox-sensitive signalling pathways where superoxide anions act as second messengers. Antioxidants inhibit redox-active signalling by blocking radical producing enzymes.³³ Thus, the antioxidant potential of this *Senecio asperulus* could be responsible for the observed anti-inflammatory activity.

Senecio asperulus methanol and aqueous extracts exhibited anti-inflammatory potential, which was moderate when compared with that of the positive control, resveratrol. All *Gunnera perpensa* extracts showed no detectable inhibition, even at the highest concentration of 200 µg/ml. This reported lipopolysaccharide induced anti-inflammatory activity assay was carried out simultaneous with the evaluation of cell viability (MTT assay) to confirm the absence of cytotoxicity of the test sample.

Cell viability

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT. The MTT assay is a colorimetric assay is used to reflect the number of viable cells present through the assessment of cell metabolic activity after treatment with test compounds. MTT, a yellow tetrazole, is reduced to purple formazan in living cells.³⁴ A solubilization

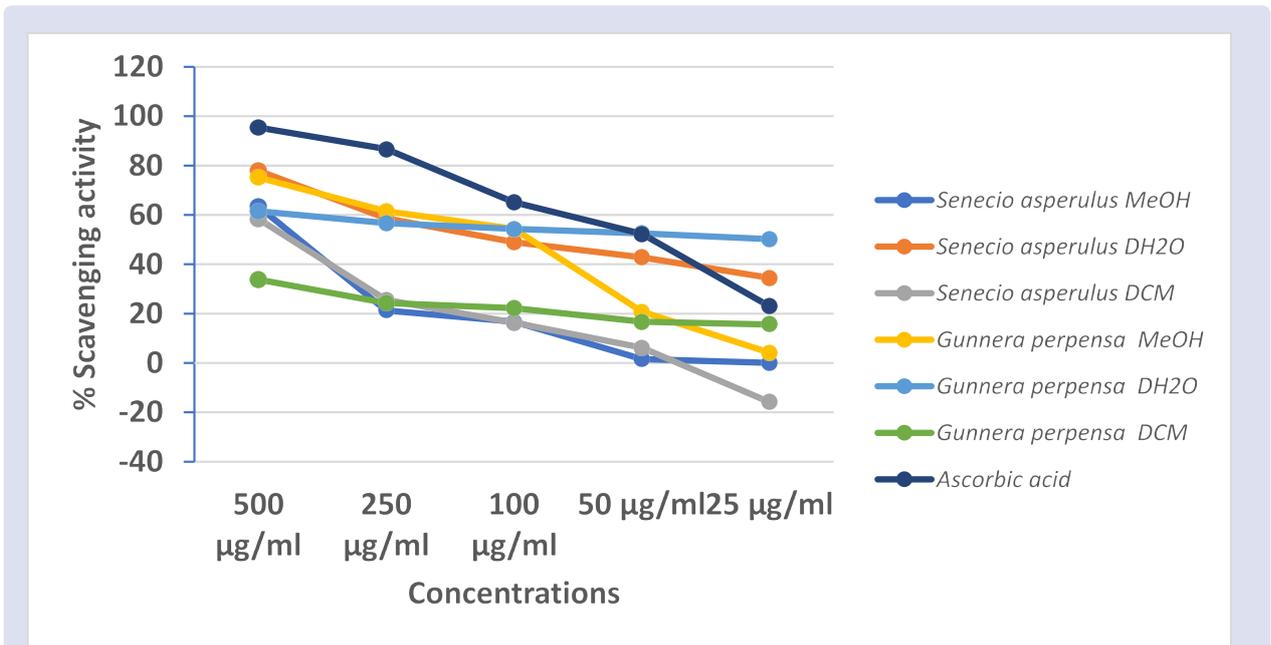


Figure 1: Antioxidant activity with DPPH assay.

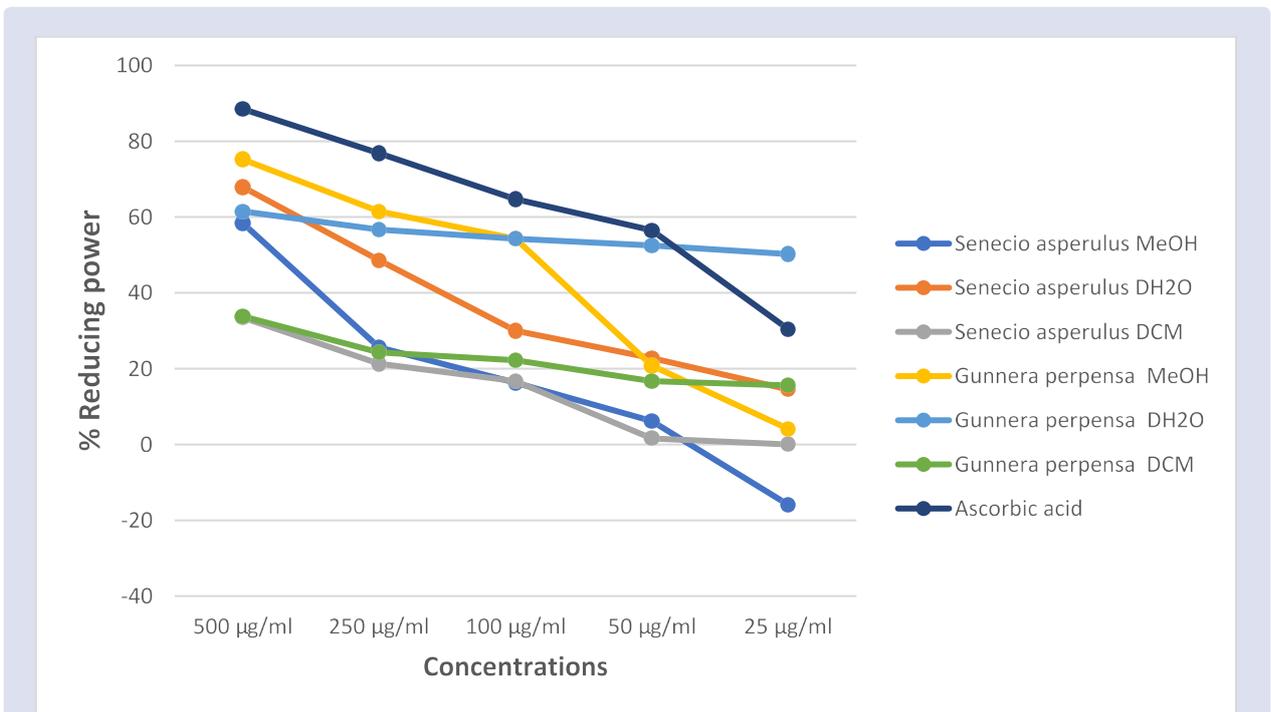


Figure 2: Ferric reducing power of *Senecio asperulus* and *Gunnera perpensa* extracts compared with ascorbic acid.

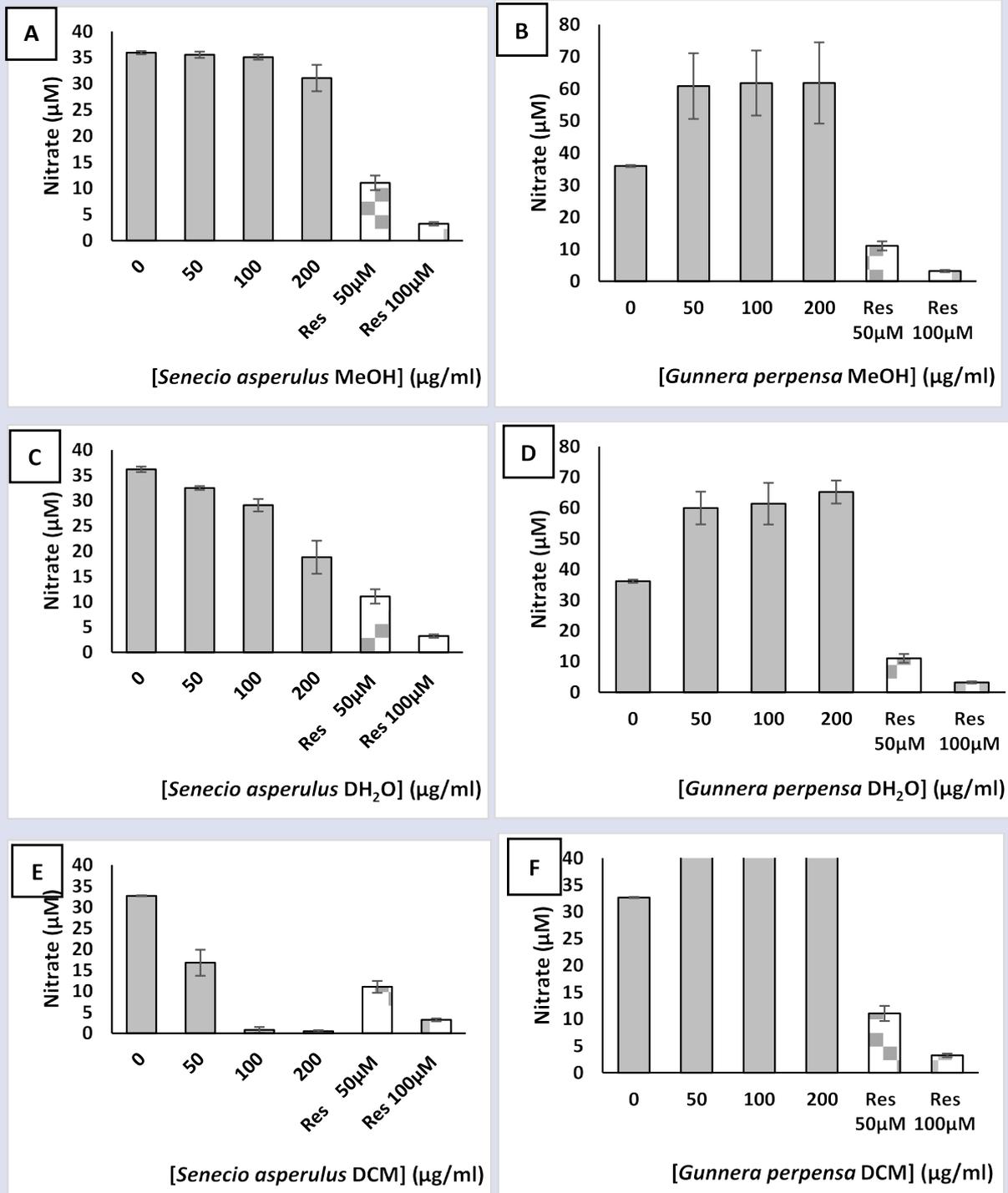


Figure 3A-F: Nitric oxide production in LPS activated macrophages treated with different concentrations of *Senecio asperulus* and *Gunnera perpensa* extracts compared with resveratrol.

solution is then added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution is quantified spectrophotometrically by measuring light absorbance at a wavelength of 600 nm. The degree of light absorption depends on the solvent. NAD(P)H-dependent oxidoreductase enzymes in the cytosolic compartment of the cell influences the reduction of tetrazolium dye.³⁵ Thus, MTT reduction depends on the cellular metabolic activity due to NAD(P)H flux.

Methanol and aqueous extracts of *Senecio asperulus* showed no significant toxicity towards RAW 264.7 cells (Figures 4A and 4C). However, the toxicity of the DCM extract of *Senecio asperulus* did override the meaningful prediction of anti-inflammatory potential from Figure 3E. Thus, re-evaluation at lower concentrations below 50 µg/ml may provide more conclusive decision however toxicity remains a potential risk for continual use of any medicine, not excluding natural medicinal plants.

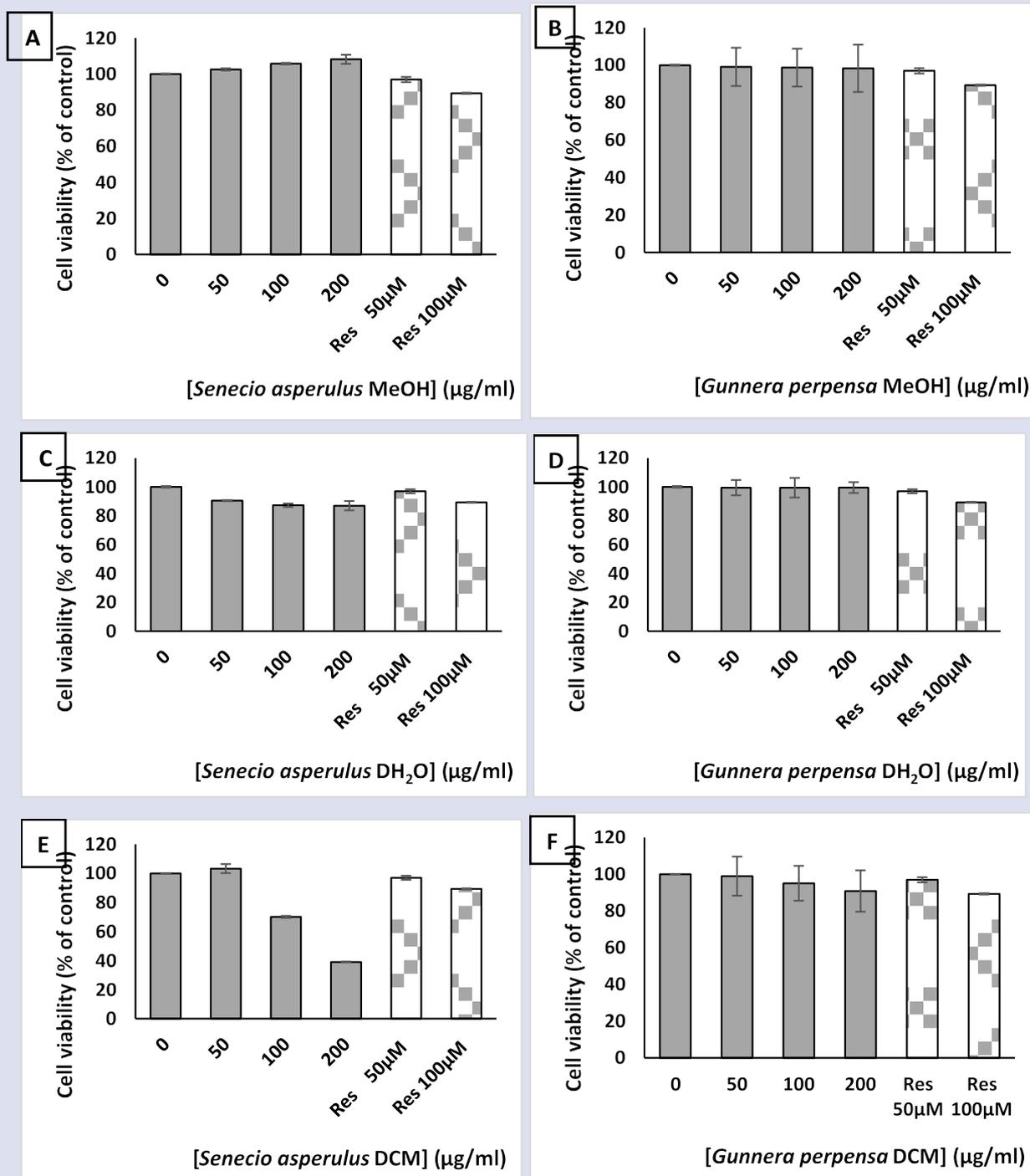


Figure 4A-F: Cytotoxicity evaluation of *Senecio asperulus* and *Gunnera perpensa* different extracts.

CONCLUSION

This study demonstrated that, *Senecio asperulus* and *Gunnera perpensa* are both rich in phytochemicals especially flavonoids, tannins, saponins and alkaloids. These phytochemicals are known for their pharmacological activities such as anti-oxidant, antidiabetic, antibacterial, antiviral, anti-inflammatory, anti-diarrheal, hepatoprotective and radioprotective effects, amongst others. Thus, based on our results, we suggest that *Senecio asperulus* aqueous and methanol extracts can be good candidates for potent anti-inflammatory, multi-drug resistant bacteria and novel antioxidant drugs development, as they had no cytotoxicity effects on tested cells. Furthermore, our results validated the Basotho traditional medicinal use of *Senecio asperulus* and *Gunnera perpensa* for the treatment of inflammation related conditions and for the treatment of wounds (due to their antimicrobial, antioxidant and anti-inflammatory activity). However, further studies for the purification of bioactive compounds (so as to synthesise these active compounds in order to promote their conservation) and *in vivo* studies, are recommended for the evaluation of these active extracts as effective anti-inflammatory, antibacterial and antioxidant agents.

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CONFLICTS OF INTEREST

No conflicts of interest to be declared by authors.

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ABBREVIATIONS

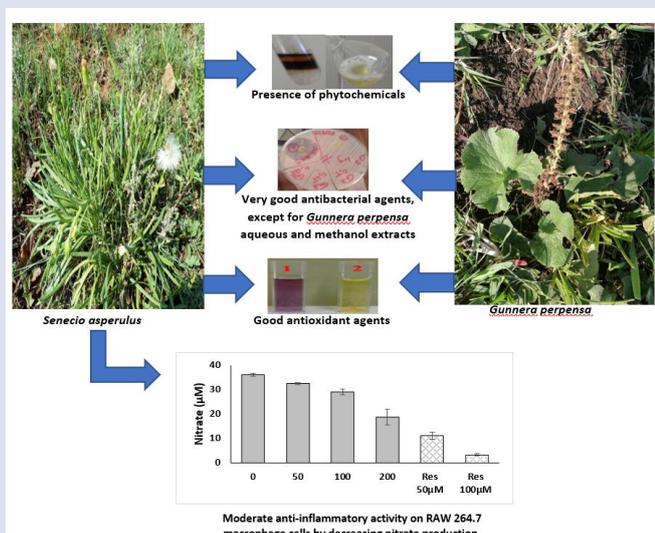
µL: Microlitre; µM: Micromolar; ATCC: American Type Culture Collection; CO₂: Carbon dioxide; COX-2: Cyclooxygenase 2; DCM: Dichloromethane; DMSO: Dimethyl sulphoxide; DNA: Deoxyribonucleic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC₅₀: Half maximal effective concentration; IF-γ: Interferon gamma; IL-1: interleukin-1; iNOS: induced nitric oxide synthesis; LP: lipoxxygenase; LPS: lipopolysaccharides; MeOH: Methanol; MIC: minimum inhibition concentration; mM: Millimolar; MTT: 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide; nm: Nanometre; NOS: Nitric oxide; NSAIDs: Non-steroidal anti-inflammatory drug; ROS: reactive oxygen species; TCA: Trichloroacetic acid; w/v: Weight per volume.

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GRAPHICAL ABSTRACT



SUMMARY

The antibacterial and antioxidant activities observed from *Senecio asperulus* aqueous extracts and *Gunnera perpensa* dichloromethane extracts related with the phytochemical contents observed from these extracts as well as the anti-inflammation activity of *S. asperulus*. To the best of the author's knowledge, there was no report on the anti-inflammation ability of *S. asperulus* roots extracts on lipopolysaccharide induced RAW 264.7 macrophages. This research study further provides evidence that antioxidants do suppress iNOS expression and subsequently nitric oxide production, thus inhibit inflammation. Moreover, these findings support the ethnomedicinal use of *Senecio asperulus* for the management of inflammation related diseases.

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