Anti-inflammatory and anticholinesterase activity of six flavonoids isolated from *Polygonum* and *Dorstenia species*

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Abstract

This study was aimed at investigating the anti-inflammatory and anticholinesterase activity of six naturally occurring flavonoids: (-) pinostrobin **(1)**, 2',4'-dihydroxy-3',6'-dimethoxychalcone **(2)**, 6-8-diprenyleriodictyol **(3)**, isobavachalcone **(4)**, 4-hydroxylonchocarpin **(5)** and 6-prenylapigenin **(6).** These compounds were isolated from *Dorstenia* and *Polygonum* species used traditionally to treat pain. The anti-inflammatory activity was determined by using the Griess assay and the 15-lipoxygenase inhibitory activity was determined with the ferrous oxidation-xylenol orange assay. Acetylcholinesterase (AChE) inhibition was determined by the Ellman's method. At the lowest concentration tested (3.12 µg/mL), compounds **2**, **3** and **4** had significant

NO inhibitory activity with 90.71%, 84.65 and 79.57% inhibition respectively compared to the positive control quercetin (67.93%). At this concentration there was no significant cytotoxicity against macrophages with 91.67%, 72.86 and 70.86% cell viability respectively, compared to 73.1% for quercetin. Compound **4** had the most potent lipoxygenase inhibitory activity (IC_{50} of 25.92 µg/mL). With the exception of (-) pinostrobin (**1**), all the flavonoids had selective anticholinesterase activity with IC_{50} values ranging between 5.93 and 8.76 µg/mL compared to the IC_{50} 4.94 µg/mL of eserine the positive control. These results indicate that the studied flavonoids especially isobavachalcone are potential anti-inflammatory natural products that may have the potential to be developed as therapeutic agents against inflammatory conditions and even Alzheimer's disease.

Key words: Flavonoids, 15-lipoxygenase, nitric oxide production inhibition, anticholinesterase

1- Introduction

Inflammation is a complex biological response of vascular tissues to infection by pathogens, damaged cells or irritants. This usually leads to the release of a variety of inflammatory mediators that in turn induce enzyme synthesis including inducible nitric oxide synthase (iNOS) and lipoxygenase (LOX). This leads to high concentrations of inflammatory nitric oxide (NO) and leukotrienes (Brain and Williams. 1990). Nitric oxide and its oxidation products are toxic and pro-inflammatory (Guzik, et al. 2003). Leukotrienes are potent eicosanoid lipid mediators derived from phospholipase-released arachidonic acid that are involved in the pathogenesis of inflammatory disorders. LOXs are the key enzymes in the biosynthesis of leukotrienes from fatty

acids producing inflammatory lipid mediators, therefore provoking inflammation-related diseases (Nathan. 1992)). Chronic inflammation increasingly appears to be involved in the onset and development of neurodegenerative diseases such as Alzheimer's disease (AD) (Akiyama, et al. 2000). Furthermore, inflammatory processes are among the pathological features associated with the central nervous system in AD (Tabet. 2006). One of the most promising approaches for treating this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase (AChE) inhibitors and there is evidence that acetyl cholinesterase (AChE) inhibitors also have an anti-inflammatory potential (Tabet. 2006). Therefore, compounds with anti-inflammatory activity could have beneficial effects on the pathophysiology of AD. For this reason, targeting inhibitors of AChE that modulate pro-inflammatory enzymes may be useful in the management of inflammatory disorders and AD. Many plant species contain compounds that might serve as leads for the development of new drugs. Some species of *Dorstenia* are traditionally used in African and South American folk medicine in the treatment of various illnesses including inflammatory and pain disorders such as arthritis, rheumatism and headache (Bouquet. 1969; Adjanohoun et al. 1996). Some of *Dorstenia* and *Polygonum* species have anti-inflammatory and anti-cholinesterase activity (Omisore, et al. 2004; Mazid, et al. 2010; Bakthir, et al. 2011; Ayaz, et al. 2014). Much of the therapeutic activity of plants may be due to their biologically active polyphenolic substances, mostly flavonoids (Di Carlo, et al. 1999). Previous studies indicated that the African *Dorstenia* and *Polygonum* species produce a variety of chalcones, flavanones, and flavones (Dzoyem, et al. 2012; Ngadjui, et al. 2000; Ngadjui, et al. 2002). Flavonoids and their precursor hydroxychalcones, are substances with many interesting biological properties including anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory and antithrombotic activities (Kumar and Pandey. 2013). Although, many flavonoids compounds

have been studied for various inflammation-related activities, a large percentage of the estimated 4,000 known flavonoids are yet to be investigated for their pharmacological activities. Much work remains to be done in order to achieve definitive conclusions about their potential involvement in different inflammatory pathways. In our continued search for biologically active compounds from plants, we investigated the inhibitory effect of three flavonoids and three chalcones against acetylcholinesterase, 15-lipoxygenasethe and the NO synthesis in LPS-activated RAW 264.7 macrophages.

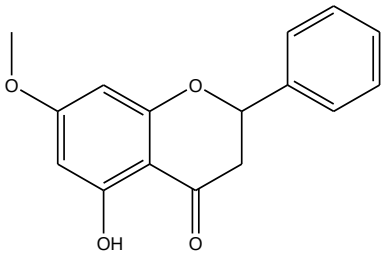
2- Materials and methods

2.1. Chemicals

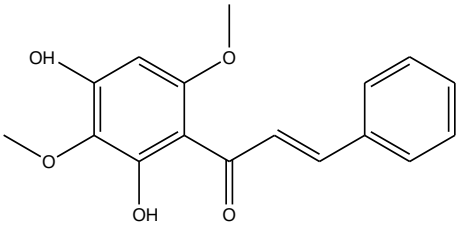
Linoleic acid was purchased from Merck (Darmstadt),. xylenol orange from Searle (England), sodium carbonate from Holpro Analytic (South Africa). Foetal calf serum (FCS), penicillin/streptomycin/fungizone (PSF) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Highveld Biological (South Africa). Phosphate buffered saline (PBS) and trypsin were purchased from Whitehead Scientific (South Africa). Quercetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). sodium dodecyl sulphate, bovine serum albumin (BSA), sodium chloride (NaCl), $MgCl_2 \cdot 6H_2O$, acetylthiocholine iodide (ATCI), eserine, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (AChE) enzyme from electric eels (type VI-S lypophilized powder), sodium nitrite, ferrous sulfate, indomethacin and 15-lipoxygenase from *Glycine max* purchased from Sigma (Germany) and. Tris(hydroxymethyl)aminomethane from Sigma, (Switzerland).

Plant materials and compounds

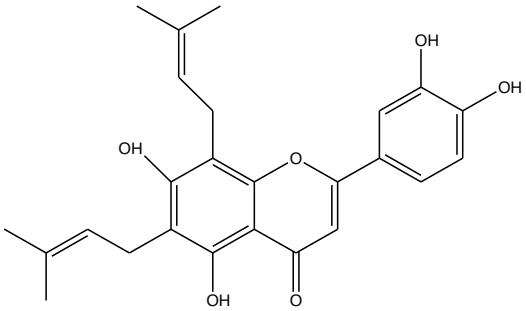
The leaves of *Polygonum limbatum* were collected in Balatchi village (Metap swampy area), near the city of Mbouda, western Region of Cameroon. The Cameroon National Herbarium, Yaoundé identified the plant (voucher specimen 38852/HNC). The twigs of *Dorstenia barteri*



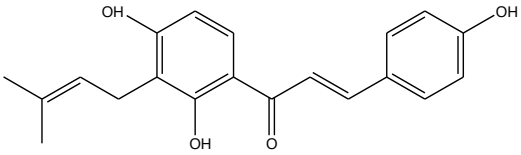
1: (-) pinostrobin



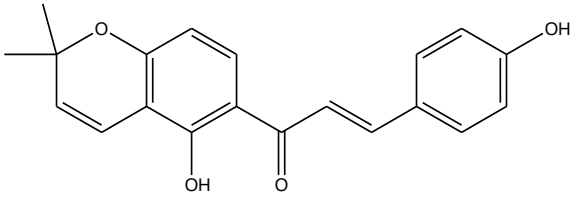
2: 2',4'-dihydroxy-3',6'-dimethoxychalcone



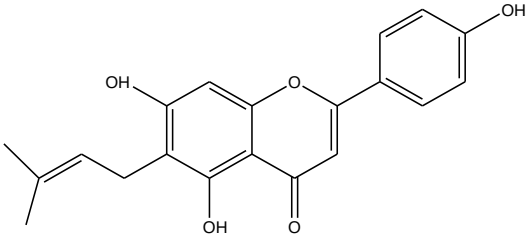
3: 6-8-diprenyleriodictyol



4: Isobavachalcone



5: 4-hydroxylonchocarpin



6: 6-prenylapigenin

Figure 1: Chemical structure of compounds

Bureau var. multiradiata were collected in Kumba, South West province of Cameroon. The National Herbarium in Yaounde identified the plantCvoucher specimen 44016/HNC).

We isolated and characterised (-) pinostrobin (**1)** and 2',4'-dihydroxy-3',6'-dimethoxychalcone **(2)** We isolated and characterised 4-hydroxylonchocarpin **(5)** and isobavachalcone **(4)** from the twigs of *D. barteri,* 6,8-diprenyleriodictyol **(3)** from the aerial parts of *D. mannii* and 6-prenylapigenin **(6)** from the twigs of *D. dinklagei*. (Ngadjui, et al. 2000; Ngadjui, et al. 2002; Ngameni, et al. 2007). The compounds were obtained from the chemical bank of the Organic Chemistry Laboratory, University of Dschang, Cameroon Chemical structures of all the compounds are shown in Fig. 1.

2.3. Anti-inflammatory activity

2.3.1. Assay of nitric oxide production and viability of LPS-activated RAW 264.7 macrophages

Cell culture

The RAW 264.7 macrophage cell lines obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in a plastic culture flask in DMEM containing L-glutamine supplemented with 10% FCS and 1% PSF solution under 5% CO_2 at 37 ^{0}C. Cells were seeded in 96 well-microtitre plates and were activated by incubation in medium containing LPS (5 µg/mL) alone (control) or LPS with different concentrations (25, 12.5, 6.25 and 3.12 µg/mL) of the compounds dissolved in DMSO. Quercetin served as a positive control NO inhibitor for the reduction of NO-production (Mu, et al. 2001).

Measurement of nitrite

Nitric oxide released from macrophages was determined by measuring the nitrite concentration in culture supernatant using the Griess reagent. After 24 h incubation, 100 µL of supernatant from each well of cell culture plates was transferred into 96-well microtitre plates and an equal volume of Griess reagent was added. The absorbance of the resultant solutions was determined on a BioTek Synergy microplate reader after 10 min at 550 nm. The concentrations of nitrite were derived from regression analysis using serial dilutions of sodium nitrite as a standard. Percentage inhibition was then calculated based on the ability of compounds to inhibit nitric oxide formation by cells compared with the control (cells in media without compounds), which was considered as 0% inhibition.

Cell viability

To determine whether the observed nitric oxide inhibition was not due to cytotoxicity, a cytotoxicity assay was also performed on the culture as previously described by Mosmann (Mosmann. 1983), with slight modifications. After removal of media, the cells were topped up with 200 µL DMEM. To each well, 30 µL of 15 mg/mL MTT were added. The cells were incubated at 37 ^{0}C in 5% CO_2. After 2 h, the medium was carefully discarded and the formed formazan salt was dissolved in DMSO. The absorbance was read at 570 nm on a BioTek Synergy microplate reader. The percentage cell viability was calculated with the control (cells without compounds containing LPS) taken as 100% viability.

2.3.2. Soybean lipoxygenase inhibition assay

The procedure of Pinto, et al. 2007, was used with slight modifications. The assay is based on the formation of the complex $Fe3^{+}$/xylenol orange with absorption at 560 nm. 15-Lipoxygenase from

Glycine max was incubated with compounds (serially diluted from 64 µg/mL to 1 µg/mL) or standard inhibitor at 25°C for 5 min. Then linoleic acid (final concentration, 140 µM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was terminated by the addition of 100 µL of FOX reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM), methanol/water (9:1)]. Quercetin and indomethacin were used as standard inhibitor and non-inhibitor of lipoxygenase respectively. For the negative control, only LOX solution and buffer were pipetted into the wells. Blanks (background) contained the enzyme LOX during incubation, but the substrate (linoleic acid) was added after the FOX reagent. The IC_{50} values of compounds leading to 50% inhibition were calculated by plotting the percentage inhibition against flavonoid concentration.

2.4. Acetylcholinesterase inhibition assay

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method (Ellman, et al. 1961), with some modifications. A mixture of 25 µL of 15 mmol/L ATCI in water, 125 µL of 3 mmol/L DTNB in Buffer A (50 mmol/L Tris-HCl, pH 8, containing 0.1 mol/L NaCl and 0.02 mol/L $MgCl_2.6H_2O$), 50 µL of Buffer B (50 mmol/L, pH 8, containing 0.1 % bovine serum albumin) and 25 µL of compound (64 µg/mL to 1 µg/mL serially diluted) we placed in wells a 96 well microplate. Then, AChE (0.2 U/mL) was added to the wells and the absorbance was determined spectrophotometrically (BioTek Synergy microplate reader) at 405 nm. Eserine (10 µg/mL starting concentration) and water were used as the positive and negative controls respectively. The IC_{50} values of compounds leading to 50% inhibition were calculated by plotting the percentage inhibition against flavonoid concentrations.

2.5. Statistical analysis

All experiments were conducted in triplicate and values expressed as mean ± standard deviation. Differences between values were assessed for significance using analysis of variance and results were compared using the Fisher's least significant difference (LSD) at 5% significance level.

3. Results and Discussion

3.1. Anti-inflammatory activity

The potential anti-inflammatory activity of flavonoids were investigated by the soybean lipoxygenase inhibition assay and by determining the amount of nitric oxide released from RAW 264.7 murine macrophage cells.

Nitric oxide production and viability of LPS-activated RAW 264.7 macrophages

In the NO production assay, cells were stimulated with 5 µg/ml lipopolysaccharide (LPS) and the effect during a co-incubation period of 24 h was determined. It is well-establishedthat, treatment of RAW 264.7 macrophages with LPS induces NO production. The magnitude can be determined by measuring the concentration of nitrite, a stable oxidized product of NO, in the media by a colorimetric procedure based on the Griess reaction (Min, et al. 2010). All the flavonoids tested had a concentration dependent inhibitory effect on NO production (Fig. 2 and 3). At the highest concentration (25 µg/mL) used, all the compounds led to a > 90% inhibition with the exception of compound **6** (81.30 % inhibition) (Fig. 2). Although compounds **2**, **3**, **4** and **5** had good activity against the NO production at 25 µg/mL, the inhibitory effect appeared to be due to their cytotoxicity against macrophages with the percentage of the cell viability of 3.98%, 3.95%, 3.9 % and 9.02 % respectively. At the same concentration, (-) pinostrobin (**1**) and 6-prenylapigenin (**6**) inhibited NO production (90.84% and 81.30% respectively) without significant cytotoxic effect(Figure 2). At the lowest concentration (3.12 µg/mL) tested,

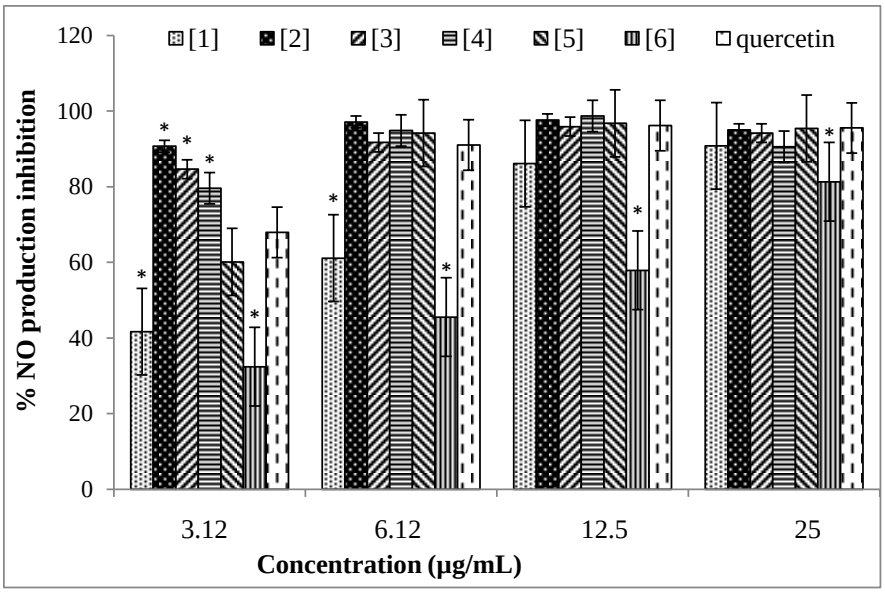


Figure 2: Inhibitory activity of flavonoids on nitric oxide production in LPS-activated RAW 264.7 macrophages after co-incubation with LPS 5 µg/mL for 24 h as determined by the % inhibition of NO production, Data represent the mean ± SD of three independent experiments $*p < 0.05$, are significantly different from the reference compound quercetin.

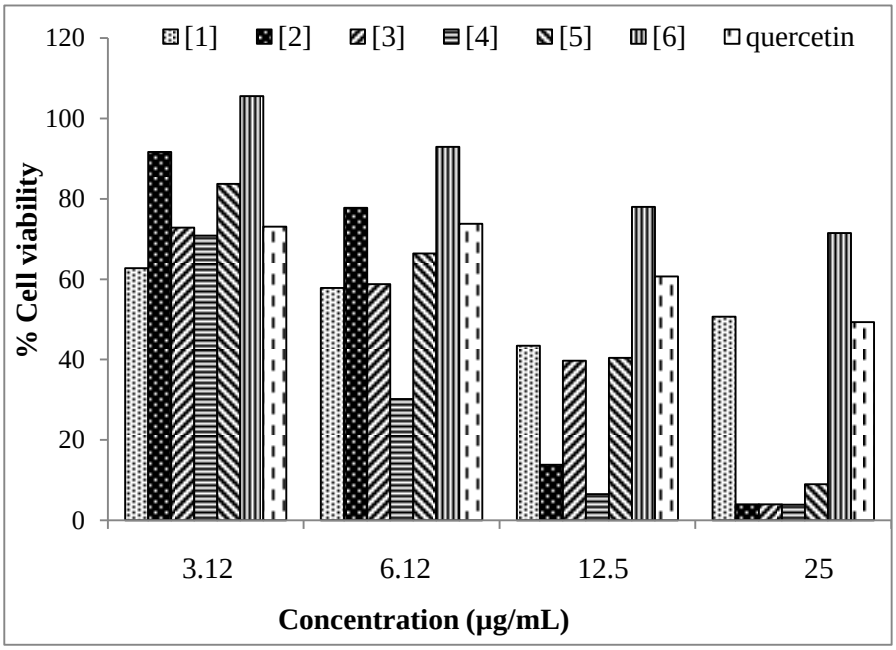


Figure 3: Influence of different concentrations of the six compounds and quercitin on % cell viability. Data represent the mean ± SD of three independent experiments.

compounds **2**, **3** and **4** had significant NO inhibitory activity compared to the positive control quercetin ($p < 0.05$). The inhibitory effect of these compounds on NO production may come from the inhibition of the activity and/or expression of iNOS enzyme (Mu, et al. 2001). Flavonoids inhibit the activity of enzymes involved in pro-inflammatory mediator's production, such as inducible nitric oxide synthase and to modify intracellular signaling pathways in immune cells (Chen, et al. 2001).

No clear structure-activity relationship was observed from our results. Compounds **2**, **4** and **5** which had the most potent activity at the lowest concentration among the six flavonoids investigated were however, all chalcones. Chalcones belong to the flavonoid family and some of them have anti-inflammatory activity. Several natural and synthetic chalcone derivatives inhibit

inducible nitric oxide synthase (iNOS)-catalyzed NO production in cell cultures (Kim, et al. 2007). Shin, et al. (2013) reported that, isobavachalcone (**4**) suppressed iNOS expression induced by macrophage-activating lipopeptide.

Soybean lipoxygenase inhibition

The ability of the six flavonoids to inhibit soybean 15-LOX activity were also determined. Compounds **3** and **4** led to > 50% inhibition (IC_{50} values 57.19 µg/mL and 25.92 µg/mL respectively). No statistically significant difference was observed between the inhibitory activity of isobavachalcone (**4**) and the positive control quercetin (IC_{50} values of 25.92 µg/mL and 25.53 µg/mL respectively). These results indicate the potential of isobavachalcone as a potent anti-lipoxygenase agent. Previous investigations have shown that different flavonoid molecules modulate the activity of arachidonic acid metabolizing enzymes such as lipoxygenase (Sadik, et

al. 2003). As expected, indomethacin had poor activity as it is known as a nonselective inhibitor of cyclooxygenase (Black, et al. 1998).

3.2. Acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) inhibitors are currently the only approved therapy for the treatment of AD. Since a large amount of evidence demonstrate that oxidative stress is intimately involved in neurodegenerative diseases, much attention have been given to flavonoids as a potential source for new natural therapeutic agents against AD (Ramassamy 2006). Therefore, flavonoids with AChE inhibitory could be promising candidates for AD treatment. Consequently we determined. the anti-AChE activity of the six compounds. All the compounds evaluated had some level of inhibitory activity against the AChE, with IC_{50} lower than 8.76 µg/mL (Table 1).

Table 1: IC_{50} (µg/ml) for 15-lipoxygenase and acetylcholinesterase inhibition by flavonoids.

Compounds and standard inhibitors	**15-LOX IC_{50} (µg/mL)**	**AChE IC_{50} (µg/mL)**
1	-*	-
2	-	6.05±0.11[a]
3	57.19±2.80[a]	6.38±0.13[a]
4	25.92±1.86[b]	5.93±0.13[b]
5	-	6.59±0.16[a]
6	45.85±0.48[c]	8.76±0.14[c]
Quercetin	25.53±1.18[c]	nd
Indomethacin	-	nd
Eserine	nd	4.94±0.05[d]

*-: samples with less than 50% inhibition, Data represent the mean ± SD of three independent experiments, values with different letters are significantly different at $p< 0.05$.

The most active compound isobavachalcone **4**, (IC_{50} of 5.93 µg/mL) had an activity close to that of the positive control eserine (IC_{50} of 4.94 µg/mL). Compound **1** had the lowest anti-AChE activity with less than 50% inhibition at the highest concentration tested (64 µg/mL). Flavonoids have previously been reported to be potent agents in combating Alzheimer's disease (AD) by enhancing acetylcholine levels. A variety of plant natural products including flavonoids and chalcones have been reported to show AChE inhibitory activity and so may be useful in the treatment of neurodegenerative disorders such as AD (Ji and Zhang 2006). To the best of our knowledge, this is the first study of the inhibitory activity of compounds **1-6** on acetylcholinesterase.

Conclusion

The overall results from this study revealed the potential of the six flavonoids compounds tested against AChE, 15-LOX or NO production. Isobavachalcone appears to be a promising multipotent agent that could possibly be developed as a therapeutic agent against inflammatory disorders and Alzheimer's disease. It is not clear whether these flavonoids would cross the blood-brain barrier and it may be interesting to investigate this aspect.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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