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in vitro Pro- and Anti-Inflammatory Screening of *Pappea capensis* Extracts

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Pappea capensis has been used traditionally in the Limpopo province of South Africa to treat different diseases caused by viruses or infectious microorganisms such as fungi and bacteria. To date no reports of pro- and anti-inflammatory activity of this plant has been reported so far. The aim of the study was to determine the intro pro- and anti-inflammatory activity of water, ethanoic and methanolic extracts of the *Pappea capensis* wood material. Serial dilutions were prepared at 0, 12.5, 25, 50, 100 and 200 µg/mL from each extract, which were subjected to the determination of the pro- and anti-inflammatory activity *in vitro* by RAW 264.7 cells using Resveratrol as a reference drug. The *in vitro* screening results for anti- and pro-inflammatory assays showed an increased nitric oxide (NO) production which correlated with each extract cytotoxicity in LPS-activated macrophages. Resveratrol, a known inhibitor of iNOS expression served as a positive control to the regime. There was a noticeable macrophage activation observed which was extracts concentrations depended. This response to treatment with different concentrations of *Pappea capensis* extracts corresponded with nitric oxide production a similar trend was observed with the analysis of cell viability which predicts the corresponding cytotoxicity of all extracts. However, there was a high nitric oxide production and observable macrophage activation in the presence of water extract A confirmatory test was done to ascertain if this effect is owed to plant constituents or due to endotoxin contamination. Then, nitric oxide production was analyzed in the presence and absence of polymyxin B (PMB) and it can be concluded that polymyxin B counteracts the pro-inflammatory effect suggesting that this activity is possibly a result of endotoxins in this wood part of the plant. The determination of endotoxin as a cofactor perpetrating pro-inflammatory activity tested with polymyxin B was used and indicated the presence of a toxin in water extract. The low anti-inflammatory effect seen in the three extracts at 200 µg/mL are not physiologically active.

Keywords: *Pappea capensis*, Pro-inflammatory activity, Anti-inflammatory activity, Resveratrol.

INTRODUCTION

Inflammation is known as the first line of defence against pathogenic effects. It can however contribute to all phases of tumorigenesis, including tumour initiation, promotion and metastasis [1]. Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation, ultimately resulting in tissue dysfunction [2,3]. Inflammatory cells can moreover secrete reactive oxygen species (ROS) that encourage mutations, lead to the failure to the DNA repair mechanisms, activation of oncogenes and eventually cancer [4]. It's of importance to understand that the cell will experience augmented genomic break, increased DNA syntheses due to lose ends, cellular proliferation, pauses in DNA repair as mentioned earlier, inhibition of apoptosis and all that will lead to promotion of angiogenesis which are normally linked with inflammation [5]. Throughout chronic inflammation, pro-inflammatory molecules such as cytokines, ROS and NFκB were secreted creating an environment that will favour exponential development of malignant cells [6]. For this reason, the anti- or pro-inflammatory activity of test samples need to be considered within the context of the disease in cancer as well as the disease progression stage at which intervention was considered, in order to accurately evaluate the potential therapeutic significance. Furthermore, multiple mechanisms may collectively contribute to an inflammatory response; consequently, it is necessary to consider that a single target specific *in vitro* model does not assess the total domain of potential therapeutic activity.

Macrophages perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis, and destruction of pathogenic microorganisms. Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents [5]. Furthermore, other diseases such as HIV and ageing which are characterised by a weakened immune response as well as diseases which evade the classical immune system (cancer) may be targeted through immune modulation [7-9].

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues, including adipose tissue [10,11]. Depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs termed classical and alternative [10]. Classical activation results in a highly inflammatory phenotype and mainly occurs in response to bacterial products such as LPS and IF-γ [10]. These classically activated macrophages produce a myriad of pro-inflammatory signals, which can alter the functionality of its surrounding cells [12]. 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) [11,13].

The mouse macrophage cell line, RAW 264.7, is a well characterized and popular model to investigate the anti-inflammatory potential of test samples [11,13]. Cells are cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation [11,13,14]. Changes in nitric oxide production are deter-mined by measuring the levels of nitrate in the culture medium [13]. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample [15].

EXPERIMENTAL

The plant material (*Pappea capensis*) was authenticated by Dr. Zietsmann Bloemfontein Museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverized by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360 g wood samples were weighed out for the extraction with 1080 mL acetone, water or methanol. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added and solutions allowed to seep out for 24 h. Filtering was performed after 24 h, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50 mL conical tubes.

Removal of solvents: Most extracts contained both aqueous and organic solvents and we employed both freeze-drying steps with a virtis freeze drier to remove aqueous solvent as well as a rotary evaporate (55 ºC) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

Preparation of sample: Extracts were solubilized in DMSO to a final concentration of 100 000 µg/mL and further diluted into culture medium as indicated in the figures. Resveratrol at 25 µM and 50 µM was used as a positive control to indicate anti-inflammatory activity.

Anti-inflammatory screening protocol: RAW 264.7 cells were seeded into 96-well plates at a density of 25,000 cells per well and allowed to attach overnight. The following day spent culture medium was removed and the samples (diluted in DMEM complete medium) added to give final concentrations of 12.5 and 50 µM (50 µL per well at double the desired final concentration). To assess the anti-inflammatory activity, 50 µL of LPS containing medium was added to the corresponding wells. Resveratrol, a known inhibitor of iNOS expression served as a positive control. Cells were then returned to the incubator for a further 20 h. To quantify nitric oxide production, 50 µL of spent culture medium was transferred to a new 96-well plate and 50 µL Griess reagent added. Absorbance was measured at 540 nm, and the results expressed relative to the appropriate untreated control. To confirm the absence of toxicity as a contributory factor and then cell viability was assessed using MTT.

Pro-inflammatory (macrophage activation) screening protocol: The same method as described above was carried out for pro-inflammatory analysis, except that LPS was not added to the treatment regime. To ensure that the effects of the plant extracts were not due to endotoxin contamination, NO production was evaluated in the presence and absence of polymyxin B (PMB) [16].

RESULTS AND DISCUSSION

The *in vitro* screening results for anti- and pro-inflammatory assays for *P. capensis* are shown in Figs. 1 and 2, respectively. Fig. 1 shows the results of NO production (A) and (B) indicates the lipopolysaccharide (LPS) activated macrophages treated with different concentrations of *P. capensis* extracts and corresponding cytotoxicity. Resveratrol, a known inhibitor of iNOS expression served as a positive control and LPS was added to the regime. In Fig. 2, macrophage activation is shown in (A), while (B) clearly shows the response to treatment with different concentrations of *P. capensis* extracts and their corresponding cytotoxicity. Also, LPS was added to the regime. Following the observatory of macrophage activation seen with water extract no. 7, there was a need to perform a confirmatory test to ensure that the effects of the plant extracts were not due to endotoxin contamination, so NO production was evaluated in the presence and absence of polymyxin B (PMB) as shown in Fig. 3.

The anti-inflammatory potential of all *P. capensis* extracts were determined using in vitro model RAW 264.7. It was found that all extracts produced high NO at low concentration and slightly reduced at 200 µg/mL as compared to resveratrol (positive control). Sharma *et al*. [14] explained the role of nitric oxide as a signalling molecule that is considered as a pro-inflammatory mediator that induces inflammation. The very low anti-inflammatory activity showed at 200 µg/mL by the three extracts cannot be considered physiologically active.

The ethanoic and methanolic extracts were not found to induce nitrate production during macrophage activation as

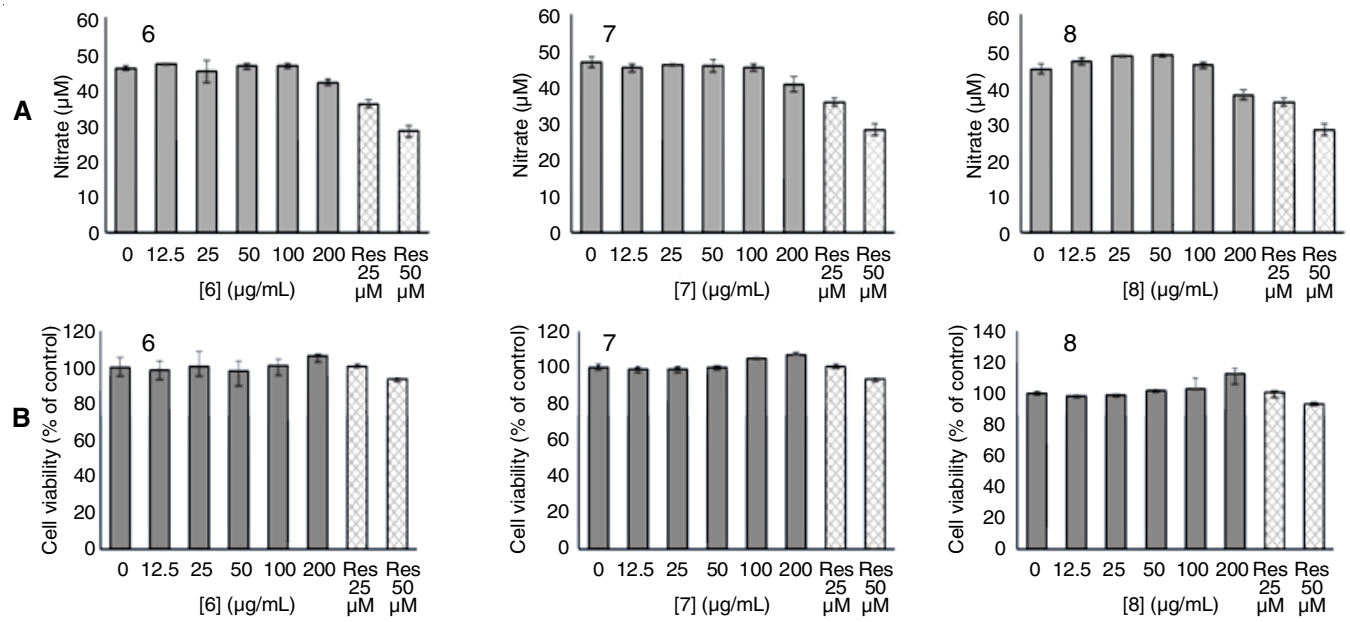

Fig. 1. Production of nitric oxide (a) in LPS activated macrophages treated with different concentrations of extracts and corresponding cytotoxicity (b)

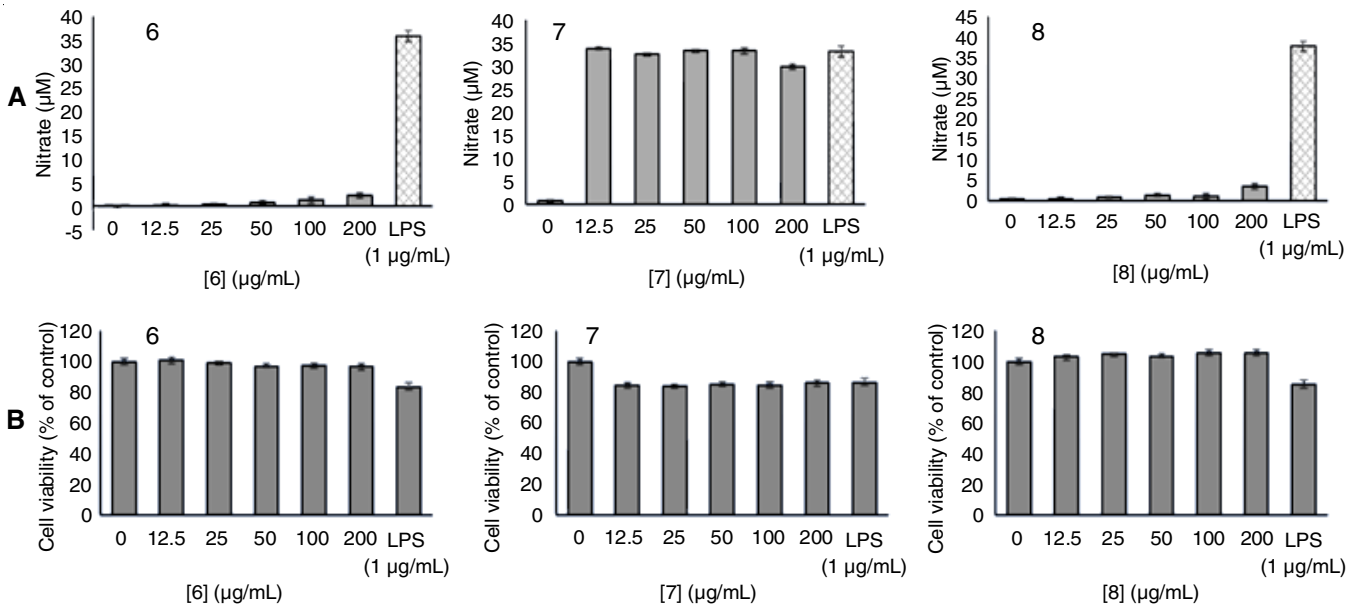

Fig. 2. Macrophage activation (a) in response to treatment with different concentrations of extracts 6, 7 and 8 and their corresponding cytotoxicity (b)

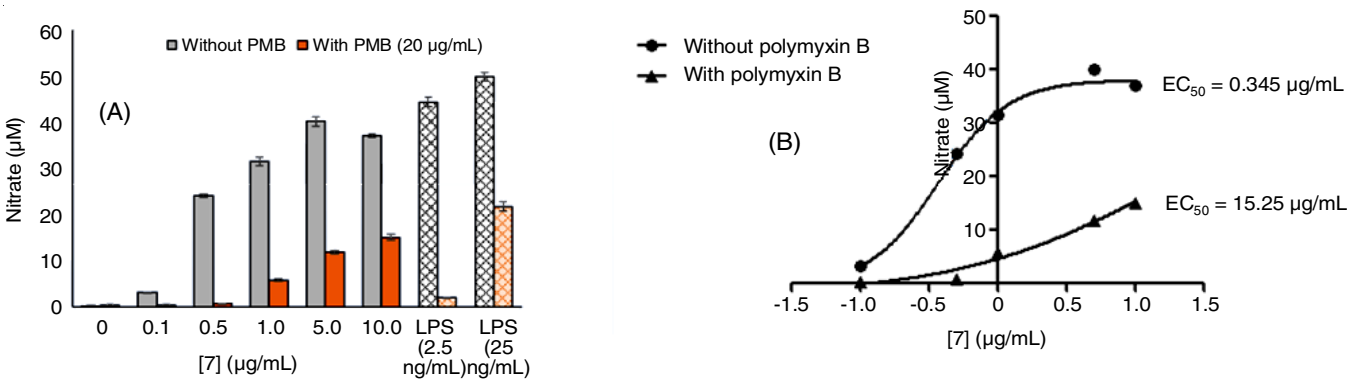

Fig. 3. Determination of endotoxin contamination using polymyxin B (PMB)

shown in Fig. 2a. It is interesting to note that the water extract which was not found to have anti-inflammatory activities was found to have elevated fluctuating none concentration dependent NO production. Water extracts results correlates with the study of Ligacheva *et al*. [17] where they found that NO production by mouse macrophages on Betula leaves contributed to the signalling of molecules to macrophage activation. None of the extracts were cytotoxic against RAW 264.7 macrophages cells as indicated in Fig. 1b and Fig. 2b.

A confirmatory test to water extract no. 7 (Fig. 2a) was done in order to ascertain whether this pro-inflammatory activity is as a result of possible endotoxin contamination of the extract with using polymyxin B. From Fig. 3, it is evident that PMB counteracts the pro-inflammatory effect suggesting that this activity is possibly a result of endotoxins in the extract, either from the solvent of extraction or as part of the plant. The determination of endotoxin as a co-factor perpetrating pro-inflammatory activity correlates with the study of Cooperstock [16], who clearly showed that polymyxin B can be used to inactivate endotoxin produced by organisms. More extensive analysis could be conducted to determine exact active compounds associated with pro-inflammatory effect because the solvent in this case used was water. The low anti-inflammatory effect seen of three extracts at 200 µg/mL are not physiologically active. Therefore, the extracts do not possess anti-inflammatory properties. Possible toxin identified in Fig. 3b could be identified by compound separation using GC-MS, but water cannot be a contributing factor harbouring a toxin. The water extract appeared to be less safe for human use.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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