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Anti-inflammatory, antioxidant, anti-tyrosinase and phenolic contents of four *Podocarpus* species used in traditional medicine in South Africa

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ABSTRACT

Ethnopharmacological relevance: Species of *Podocarpus* are used traditionally in their native areas for the treatment of fevers, asthma, coughs, cholera, chest complaints, arthritis, rheumatism, venereal diseases and distemper in dogs.
Aims of the study: To investigate the antioxidant, anti-inflammatory and anti-tyrosinase activities of four *Podocarpus* species, *Podocarpus elongatus*, *Podocarpus falcatus*, *Podocarpus henkelii* and *Podocarpus latifolius*, used in traditional medicine in South Africa. Phytochemical analysis to determine the phenolic contents was also carried out.
Materials and methods: DPPH, FRAP and β-carotene-linoleic acid assays were used to determine the antioxidant/radical scavenging activities of these species. Anti-inflammatory activity of these species was assayed against two cyclooxygenase enzymes (COX-1 and COX-2). Tyrosinase inhibition activity was analysed using the modified dopachrome method with L-DOPA as the substrate. Phenolics were quantitatively determined using spectrophotometric methods.
Results: Stems of *Podocarpus latifolius* exhibited the lowest EC_{50} (0.84 μg/ml) inhibition against DPPH. The percentage antioxidant activity based on the bleaching rate of β-carotene ranged from 96% to 99%. High ferric reducing power was observed in all the extracts. For COX-1, the lowest EC_{50} value was exhibited by stem extracts of *Podocarpus elongatus* (5.02 μg/ml) and leaf extract of *Podocarpus latifolius* showed the lowest EC_{50} against COX-2 (5.13 μg/ml). All extracts inhibited tyrosinase activity in a dose-dependent manner with stem extract of *Podocarpus elongatus* being the most potent with an EC_{50} value of 0.14 mg/ml. The total phenolic content ranged from 2.38 to 6.94 mg of GAE/g dry sample.
Conclusion: The significant pharmacological activities observed support the use of these species in traditional medicine and may also be candidates in the search for modern pharmaceuticals in medicine, food and cosmetic industries.

1. Introduction

Podocarpus species are used in traditional medicine in their native areas (Abdillahi et al., 2010). These species are used to treat a number of ailments including fevers, asthma, coughs, cholera, distemper, chest complaints, arthritis, rheumatism, painful joints and venereal diseases (Duke and Ayensu, 1985; Chopra et al., 1986; Riley, 1994). Four species of *Podocarpus* occur in South Africa; *Podocarpus elongatus* L' Hèrit., *Podocarpus falcatus* (Thunb.) R. Br. Ex Mirb., *Podocarpus henkelii* Stapf ex Dallim. & Jacks. and *Podocarpus latifolius* (Thunb.) R. Br. Ex Mirb. (Barker et al., 2004). These species are used in traditional medicine mainly in KwaZulu-Natal and the Eastern Cape (Hutchings et al., 1996; Masika and Afolayan, 2003). The sap from these four species was used by woodmen working in southern African forests to treat chest complaints (Watt and Breyer-Brandwijk, 1962). In the Eastern Cape, the bark of *Podocarpus falcatus* and *Podocarpus latifolius* is used as a decoction to treat gall sickness in cattle and distemper in dogs (Dold and Cocks, 2001; Masika and Afolayan, 2003). Hutchings et al. (1996) mentioned that the bark of *Podocarpus henkelii* and *Podocarpus latifolius* is widely used in Zulu traditional medicine, though the uses are not indicated. *Podocarpus falcatus* and *Podocarpus latifolius* are also used in traditional medicine in other African countries. The Maasai of Kenya use bark decoction as a remedy for stomach ache (Beentje, 1994). *Podocarpus falcatus* and *Podocarpus latifolius* are used to treat

Abbreviations: BHT, butylated hydroxytoluene; COX, cyclooxygenase; L-DOPA, L-3,4-dihdroxyphenylalanine; DOPA, dopamine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Fe (III)-ADP/NADH, iron-3-adenosine disphosphate/nicotinamide dinucleotide-oxidase; Fe (III)-ADP/NADPH, iron-3-adenosine disphosphate/nicotinamide adenine dinucleotide phosphate-oxidase; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; mRNA, messenger ribonucleic acid.

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cattle diseases and stomach ache in East Africa (Sindiga, 1995). Oils from *Podocarpus falcatus* are used for treating gonorrhoea and bark is used for curing headaches in Ethiopia (Pankhurst, 2000).

Phytochemical and pharmacological studies on a number of *Podocarpus* species have led to the isolation of diterpenoids which exhibit antioxidant activity by inhibiting microsomal and mitochondrial lipid peroxidation induced by Fe (III)-ADP/NADH (Haraguchi et al., 1997). Totarol, a compound isolated from many *Podocarpus* species inhibit linoleic acid autooxidation, mitochondrial and microsomal lipid peroxidation induced by Fe (III)-ADP/NADPH. This compound protects red blood cells against oxidative hemolysis and mitochondrial respiratory enzyme activities against NADPH induced oxidative injury (Haraguchi et al., 1997). Totarol is now commercially produced from *Podocarpus totara* as Totarol™, and is effective as a topical anti-inflammatory agent (Gendimenico, 2005). Biflavonoids isolated from *Podocarpus macrophyllus* are known to regulate pro-inflammatory gene expression both *in vitro* and *in vivo*, and also exhibit phospholipase A_2 and cyclooxygenase-2 inhibitory activity (Kim et al., 2008). A flavonoid (2,3-dihydro-4′,4‴di-*O*-methylamentoflavone) isolated from *Podocarpus macrophyllus* exhibited potent tyrosinase inhibitory activity at 0.1 mM (53.2% inhibition). Varying concentration of this compound (0.04–0.1 mM) strongly inhibit the expression of tyrosinase related protein-2 (TRP-2) by decreasing both protein and mRNA levels (Cheng et al., 2007). Other than their traditional uses, chemotaxonomy, phytochemistry, phylogeny and pharmacological approaches based on a review done on the genus *Podocarpus* (Abdillahi et al., 2010) were used in selecting the South African species for screening. Based on the literature search carried out, no scientific reports have been published on the potential antioxidant, anti-inflammatory and anti-tyrosinase activities of South African *Podocarpus* species.

2. Materials and methods

2.1. Plant material

Leaves and young stems of *Podocarpus elongatus*, *Podocarpus falcatus*, *Podocarpus henkelii* and *Podocarpus latifolius* were collected from the National Botanical Garden, Pietermaritzburg. Identification of the plant material was done at the University of KwaZulu-Natal Herbarium with the help of the taxonomist in-charge Dr. Christina Potgieter. Voucher specimens (HA 001NU, HA 002NU, HA 003NU and HA 004NU), were deposited at the University of KwaZulu-Natal Herbarium. Plant material used in the screening were dried at room temperature and then ground into fine powders. Fifty percent methanol (50% MeOH) was used for extracting plant material for antioxidant and anti-tyrosinase activity. Here, 100 ml of 50% MeOH was added to 5 g of dried plant material, then sonicated for 1 h in an ultrasound bath, filtered under vacuum using Whatman No. 1 filter paper. Three solvents: petroleum ether (PE), dichloromethane (DCM), and 80% ethanol were used for sequential extraction of plant materials for anti-inflammatory activity. A hundred millilitres of PE was added to 5 g of dried material first, then sonicated for 1 h in an ultrasound bath, kept overnight and then filtered under vacuum using Whatman No. 1 filter paper. This was then followed with DCM, and finally 80% ethanol. The clear filtrates were dried under vacuum using a rotary evaporator. Percentage yield of all plant extracts were obtained (Table 1).

Table 1
Percentage yield of plant extracts prepared from dried plant material of four South African *Podocarpus* species.

Podocarpus species	Plant part	PET	DCM	Ethanol	Methanol
Podocarpus elongatus	Leaves	3.9	1.6	3.8	2.8
	Stem	1.1	0.9	4.9	1.1
Podocarpus falcatus	Leaves	1.5	2.1	2.3	5.9
	Stem	2.6	0.9	4.1	3.1
Podocarpus henkelii	Leaves	2.5	3.1	2.3	1.4
	Stem	0.8	1.8	1.9	1.2
Podocarpus latifolius	Leaves	4.1	3.1	2.4	3.2
	Stem	3.3	0.8	2.4	2.2

2.2. Antioxidant activity

The free-radical scavenging activity of plant extracts were measured using the DPPH assay as described by Karioti et al. (2004) with slight modifications. The plant extracts were prepared at varying concentrations (0.065, 0.26, 0.52, 1.04, 6.25, 12.5 mg/ml) giving final reaction mixture concentrations of 0.65, 2.6, 5.2, 10.4, 65 and 125 μg/ml, respectively. Ascorbic acid and BHT (5–100 μM) were used as positive controls. Methanolic DPPH solution (0.1 mM) was used as a negative control. Fifteen microlitres of the plant extracts and the controls were diluted with 735 μl of methanol. This was added to 750 μl of 0.1 mM of the methanolic DPPH solution, giving a final volume of 1.5 ml. After incubation at room temperature, in the dark for 30 min, the absorbance was read in a spectrophotometer (Varian Cary) at 517 nm. The absorbance of extracts without DPPH was measured and subtracted from the corresponding reading with DPPH. This was done in order to correct the absorbance due to the colour of the extracts. The extracts and the controls were tested in triplicate. The free-radical scavenging activity (RSA) of the extracts against DPPH was calculated according to the formula:

$$\%\ \ \mathrm{RSA} = 100 \times \left(1 - \frac{A_E}{A_D}\right),$$

where A_E is the absorbance of the standard antioxidant or extract, and A_D is the absorbance of the negative control.

The ability of plant extracts to prevent or minimize the coupled oxidation of β-carotene and linoleic acid in an emulsified aqueous system (Parejo et al., 2002) was measured according to the method described by Amarowicz et al. (2004) with modifications. Initially, 10 mg of β-carotene was dissolved in 10 ml chloroform in a brown Schott bottle. The chloroform was evaporated in a desiccator leaving a thin film of β-carotene, 200 μl of linoleic acid and 2 ml of Tween 20 were immediately added to the β-carotene and mixed with aerated distilled water (497.8 ml), giving a final β-carotene concentration of 20 μg/ml. This mixture was further saturated with oxygen by vigorous agitation to form an orange coloured emulsion. From the emulsion, 4.8 ml was dispensed in test tubes and 200 μl of BHT and plant extract (6.25 mg/ml) were added, giving a final concentration of 250 μg/ml in the reaction mixtures. The absorbance was measured at $t = 0$ and the reaction mixtures were incubated for 2 h at 50 °C. The absorbance of each reaction mixture was subsequently measured every 15 min for 120 min at 470 nm. A Tween 20 solution was used to blank the spectrophotometer. Fifty percent methanol was used as a negative control and the extracts were tested in triplicate. The antioxidant activity was expressed in three different ways. First the rate of β-carotene bleaching was calculated using the following formula:

$$\text{Rate of β-carotene bleaching} = \ln\left(\frac{A_{t=0}}{A_{t=t}}\right) \times \frac{1}{t} \quad (1)$$

where $A_{t=0}$ is the absorbance of the emulsion at 0 min; and $A_{t=t}$ is the absorbance at time t (30, 60, 90 min). The average rate of β-carotene bleaching was calculated based on rates determined at 30, 60 and 90 min. The calculated average rates were used to determine the antioxidant activity (ANT) of the respective extracts, and expressed

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Table 2
DPPH radical scavenging activity (%) and EC_{50} of plant extracts of four species of *Podocarpus* used in traditional medicine in South Africa.

Podocarpus species	Plant part	Concentration of methanolic extracts (μg/ml)						
		0.65	2.6	5.2	10.4	65	125	EC_{50}
Podocarpus elongatus	Leaves	33.40	74.43	92.59	93.76	96.22	97.64	1.50 ± 0.01^{d}
	Stem	26.74	48.70	82.17	91.61	91.68	92.26	2.10 ± 0.02^{f}
Podocarpus falcatus	Leaves	42.65	51.39	89.09	92.60	92.66	94.02	0.99 ± 0.04^{b}
	Stem	39.71	56.89	81.95	94.04	94.26	95.90	1.11 ± 0.02^{c}
Podocarpus henkelii	Leaves	39.90	57.15	92.90	94.35	94.65	95.37	1.04 ± 0.04^{bc}
	Stem	41.10	72.24	89.93	93.28	93.39	95.16	1.65 ± 0.02^{c}
Podocarpus latifolius	Leaves	14.18	74.34	84.08	92.42	95.23	96.55	1.02 ± 0.06^{b}
	Stem	10.30	54.91	89.19	90.46	93.72	93.94	0.84 ± 0.01^{a}

EC_{50} for positive controls: ascorbic acid (0.55 μg/ml); BHT (5.19 μg/ml). Values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.

as percent inhibition of the rate of β-carotene bleaching:

$$\%\ \text{ANT} = \frac{(R_{\text{control}} - R_{\text{sample}})}{R_{\text{control}}} \times 100 \quad (2)$$

where R_{control} and R_{sample} represent the respective average β-carotene bleaching rates for the standard antioxidant and plant extracts, respectively. The antioxidant activity was further expressed as an oxidation rate ratio (ORR) based on the equation;

$$\text{ORR} = \frac{R_{\text{sample}}}{R_{\text{control}}} \quad (3)$$

Finally, antioxidant activity (AA) was calculated as the percentage of inhibition of coupled oxidation of β-carotene and linoleic acid against the negative control and BHT based on the absolute changes in absorbance measurements at $t = 60$ min and $t = 120$ min during the assay rather than as an average rate. In Eq. (4) below, the results are normalized by introducing two extremes; negative control which offers no protection against oxidation of the linoleic acid/β-carotene emulsion, hence the antioxidant activity is defined as 0% for this system; and the BHT control which should offer approximately 100% inhibition against oxidation over the time course of the assay. The antioxidant activity was expressed as:

$$\%\ \text{AA} = \left[1 - \frac{(A_{Et=0} - A_{Et=t})}{\{(A_{Nt=0} - A_{Nt=t}) + (A_{Bt=0} - A_{Bt=t})\}}\right] \times 100 \quad (4)$$

where $A_{Et=0}$ is the absorbance of the test extract at the beginning of incubation; $A_{Et=t}$ is the absorbance at time $t = 60$ or 120 min with the test extract; and $A_{Nt=0}$ and $A_{Nt=t}$ represent the absorbance of the negative control at the beginning of incubation and at time $t = 60$ or 120 min, respectively; $A_{Bt=0}$ is the absorbance of the synthetic antioxidant (BHT) at 0 min and $A_{Bt=t}$ is the absorbance at $t = 60$ or 120 min.

The reducing powers of the extracts were determined using the ferric reducing power (FRAP) assay, as described by Lim et al. (2009) with slight modifications. Plant extracts plus the positive controls (ascorbic acid and BHT 6.25 mg/ml) were dissolved in methanol. Thirty microlitres of each sample were added to a microplate containing 30 ml of water and serially diluted. Subsequently, potassium phosphate buffer (40 μl, 0.2 M, pH 7.2) and potassium ferricyanide (40 μl, 1%, w/v) were added. The reaction mixtures were incubated at 50 °C for 20 min and afterwards, trichloroacetic acid (40 μl, 10%, w/v), distilled water (150 μl) and $FeCl_3$ (30 μl, 0.1%, w/v) were added. These mixtures were further incubated at room temperature for 30 min in the dark. Absorbance was measured at 630 nm. The ferric reducing power capacities of the extracts and standard antioxidants were expressed graphically by plotting absorbance against concentration. Samples for the assay were prepared in triplicate. Increase in absorbance of the reaction mixtures indicates greater reducing power.

2.3. Anti-inflammatory activity

The anti-inflammatory activities of the extracts were evaluated using the enzyme-based cyclooxygenase assays: COX-1 and COX-2 (Jäger et al., 1996). Both COX-1 and COX-2 enzymes were obtained from Sigma–Aldrich (Eldeen and Van Staden, 2008). The extracts were tested at a concentration of 250 μg/ml per test solution. Indomethacin at 5 μM for COX-1 and 200 μM for COX-2 was used as a positive control, while, background (the enzyme was inactivated with HCl before addition of ^{14}C-arachidonic acid) and solvent blank were used as negative controls. For each assay, a duplicate set of samples were tested and the assay was repeated 3 times. The anti-inflammatory activity of the extracts was measured as a percentage of inhibition. This was done by determining the amount of radioactivity in the solutions relative to that of the solvent blank. The formula below was used to calculate percentage of inhibition:

$$\text{Inhibition} = \left[\frac{(\text{Radioactivity}_{\text{sample}} - \text{Radioactivity}_{\text{background}})}{(\text{Radioactivity}_{\text{blank}} - \text{Radioactivity}_{\text{background}})}\right] \times 100$$

Table 3
Antioxidant activity of extracts of *Podocarpus* species in the β-carotene–linoleic acid assay.

Plant species/control	Plant part	ANT (%)	ORR	$AA_t = 60$ min	$AA_t = 120$
Podocarpus elongatus	Leaves	99.04 ± 0.30^{a}	0.135 ± 0.010^{ab}	80.49 ± 0.82^{ab}	61.17 ± 1.50^{bc}
	Stem	99.44 ± 0.31^{a}	0.081 ± 0.001^{a}	89.10 ± 3.16^{a}	76.29 ± 2.00^{a}
Podocarpus falcatus	Leaves	96.12 ± 1.70^{a}	0.283 ± 0.010^{ab}	79.08 ± 2.75^{ab}	61.08 ± 4.18^{bc}
	Stem	99.40 ± 0.36^{a}	0.086 ± 0.001^{a}	89.77 ± 4.40^{a}	66.21 ± 3.05^{b}
Podocarpus henkelii	Leaves	98.50 ± 0.65^{a}	0.213 ± 0.001^{ab}	73.75 ± 3.09^{b}	58.45 ± 3.40^{bc}
	Stem	98.89 ± 0.65^{a}	0.159 ± 0.001^{ab}	76.82 ± 2.53^{b}	59.83 ± 1.75^{bc}
Podocarpus latifolius	Leaves	96.39 ± 3.89^{a}	0.511 ± 0.001^{b}	73.44 ± 4.33^{b}	57.45 ± 4.79^{c}
	Stem	99.21 ± 0.25^{a}	0.112 ± 0.337^{ab}	82.83 ± 3.12^{a}	60.08 ± 4.86^{bc}
BHT		97.05	0.425		

ANT = antioxidant activity based on average rate of β-carotene bleaching (at 30, 60, and 90 min); ORR = oxidation rate ratio; AA = antioxidant activity of extract at $t = 60$ and 120 min. Values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.

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The EC_{50} of the most active extracts were determined by serially diluting the extracts from 250 to 4.625 μg/ml. The diluted extracts were subjected to COX-1 and COX-2 bioassays as described above.

2.4. Tyrosinase inhibition

Tyrosinase inhibitory activity of the extracts was determined as described by Chan et al. (2008) using a modified dopachrome method with L-DOPA as the substrate. Plant extracts were dissolved in 50% DMSO at a concentration of 5 mg/ml. A 40 μl aliquot of each sample was added to 80 μl of 0.1 M phosphate buffer (pH 6.8) followed by 40 μl of tyrosinase (31 units/ml) and 40 μl of L-DOPA (2.5 mM). The samples were incubated for 30 min at 37 °C. Each sample was accompanied by a blank consisting of all components except L-DOPA. Catechin which is known to inhibit tyrosinase (No et al., 1999) was used as a positive control. Results were compared with a control and a blank containing 50% DMSO in place of the sample solution. The percentage of tyrosinase inhibition was calculated as:

$$\%\ \text{tyrosinase inhibition} = \left[\frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right] \times 100$$

2.5. Phenolic analysis

Plant materials were extracted as described by Makkar (1999). Two grams of plant material were extracted with 10 ml of 50% methanol and filtered under vacuum using Whatman No. 1 filter paper. The total phenolic (TP) content in the plant extracts was estimated using the Folin Ciocalteu (Folin C) assay, as described by Makkar (1999) with slight modifications. The results were expressed as gallic acid (GAE) equivalents. Condensed tannins (CT) were determined by using the butanol–HCl assay (Porter et al., 1986). The formula: (A_{550} nm × 78.26 × dilution factor) ÷ (% dry matter) was used to calculate condensed tannins (% per dry matter) as leucocyanidin (Leuco) equivalents. The rhodanine assay described by Makkar (1999) was used to determine the gallotannin (GT) content in the plant extracts. Gallotannin content was expressed as gallic acid (GAE) equivalents. The total flavonoid (TF) content was determined using the vanillin assay. The flavonoid content was expressed as catechin equivalents per gram of dry weight (Makkar, 1999).

2.6. Statistical analysis

Data were expressed as mean ± standard error of mean (S.E.M.). One-way analysis of variance (ANOVA) using SPSS version 10 software (SPSS Inc., Chicago, USA) and Duncan Multiple Range Test was used to determine the differences among the means. P values of 0.05 ($P = 0.05$) was regarded as significant. The EC_{50} values were calculated using a logarithmic non-linear regression curve derived from the plotted data using GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA). Linear regression analysis was used to observe the correlation between the phenolic contents and antioxidant activities.

3. Results

3.1. Antioxidant activities

The radical scavenging activity was expressed as a percentage decrease in absorbance at 517 nm. All extracts from the four species showed a significant percentage radical scavenging activity with increase in plant concentration (Table 2). Stem extract of *Podocarpus latifolius* had the lowest EC_{50} value of 0.84 μg/ml. While this was

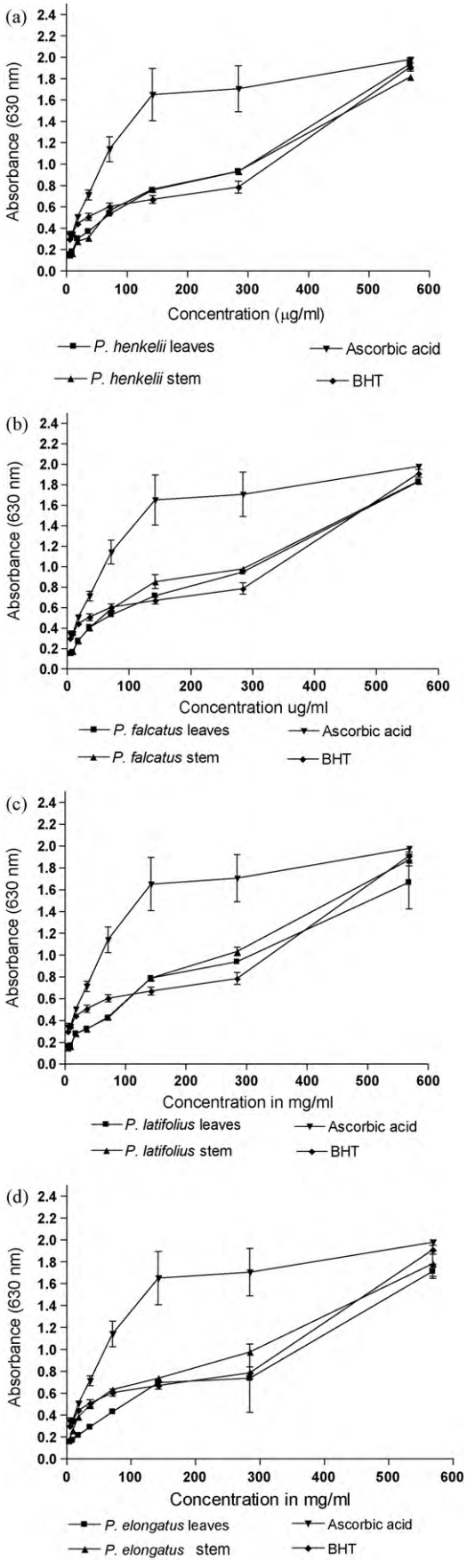

Fig. 1. Reducing powers of methanolic extracts from four species of *Podocarpus*.

Table 4
Percentage COX-1 and COX-2 inhibition of different plant extracts of *Podocarpus* species at 250 μg/ml.

Podocarpus species	Plant part	Extraction solvent					
		Petroleum ether		Dichloromethane		80% Ethanol	
		COX-1	COX-2	COX-1	COX-2	COX-1	COX2
Podocarpus elongatus	Leaves	89.79 ± 0.8^{defghi}	90.59 ± 0.5^{abcdef}	81.32 ± 6.6^{ghijkl}	81.36 ± 0.8^{fg}	75.99 ± 9.1^{jkl}	70.09 ± 6.9^{h}
	Stem	100.0 ± 1.6^{abcd}	93.24 ± 3.6^{abcd}	91.51 ± 7.3^{abcdefg}	82.36 ± 4.0^{efg}	79.15 ± 2.1^{ijkl}	62.32 ± 1.8^{hi}
Podocarpus falcatus	Leaves	97.97 ± 2.1^{abcde}	87.33 ± 1.2^{bcdefg}	82.47 ± 3.1^{ghijkl}	79.09 ± 1.8^{g}	79.85 ± 1.1^{hijkl}	68.65 ± 11.4^{h}
	Stem	102.43 ± 3.0^{a}	96.52 ± 2.6^{ab}	90.33 ± 7.1^{cdefgh}	81.69 ± 2.5^{efg}	78.16 ± 7.4^{jkl}	66.28 ± 3.9^{hi}
Podocarpus henkelii	Leaves	100.73 ± 4.3^{abc}	90.80 ± 3.6^{abcde}	88.18 ± 7.4^{efghij}	86.12 ± 2.6^{cdefg}	76.95 ± 6.8^{jkl}	70.10 ± 13.7^{h}
	Stem	101.93 ± 2.7^{ab}	94.63 ± 3.4^{abc}	86.79 ± 10.2^{fghijk}	86.78 ± 1.9^{cdefg}	73.21 ± 5.6^{l}	64.22 ± 8.9^{hi}
Podocarpus latifolius	Leaves	96.97 ± 2.1^{abcdef}	91.18 ± 2.7^{abcde}	78.14 ± 7.0^{jkl}	84.41 ± 2.8^{defg}	74.71 ± 4.5^{l}	68.84 ± 4.0^{h}
	Stem	102.43 ± 3.0^{a}	97.59 ± 1.3^{a}	91.16 ± 5.8^{bcdefg}	81.20 ± 0.8^{fg}	75.99 ± 9.15^{kl}	57.87 ± 2.3i

Values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.

higher than ascorbic acid (0.55 μg/ml), it was much lower than BHT (5.19 μg/ml) (Table 2).

The plant extracts were found to hinder the extent to which β-carotene is bleached by neutralising the linoleate-free radical and other free radicals formed in the system (Table 3). Apart from the leaf extracts of *Podocarpus falcatus* (96.12%) and *Podocarpus latifolius* (96.39%), all other extracts from the four species exhibited a higher percentage inhibition (98.50–99.44%) than the positive control (i.e. BHT) which gave a percentage inhibition of 97.05% (Table 3). The percentage antioxidant (% ANT) based on the bleaching rate of β-carotene ranged from 96.12% to 99.44% (Table 3). Stem extracts of *Podocarpus elongatus* exhibited the most potent antioxidant activity (99.44%). After normalizing the data at 60 and 120 min of incubation, the stem extract of *Podocarpus falcatus* gave the highest inhibition of 89.77% at 60 min and stem extract of *Podocarpus elongatus* gave a percentage inhibition of 76.29% at 120 min (Table 3).

Figure 1 shows the ferric reducing power of the extracts and positive controls. All extracts exhibited dose dependant reducing power with ascorbic acid giving the highest absorbance. The reducing activity of leaf extract of *Podocarpus henkelii* and stem extract of *Podocarpus latifolius* was higher than BHT, while the rest of the extracts gave slightly lower values than BHT. The most potent extract was the leaves of *Podocarpus henkelii* while the least potent was the leaves of *Podocarpus latifolius*.

3.2. Anti-inflammatory activities

Table 4 shows the inhibition of COX-1 and COX-2 enzymes by petroleum ether, dichloromethane and 80% ethanol extracts. A value of inhibition of 60% by organic solvents is considered significantly active. All extracts inhibited COX-1 and COX-2 prostaglandin biosynthesis at a concentration greater than 60% (Table 4). However, COX-2 inhibitory values were lower than for COX-1 (Table 4). For COX-1, PE stem extracts of all four species exhibited complete inhibition. PE stem extracts of *Podocarpus latifolius* and *Podocarpus falcatus* gave the highest inhibition against COX-2, 97.59% and 96.52%, respectively. Table 5 shows the concentration of the extracts required to inhibit both COX-1 and COX-2 activity by 50%. For COX-1, PE stem extract of *Podocarpus elongatus* was the most potent with an EC_{50} value of 5.02 μg/ml, while for COX-2 the leaf extract of *Podocarpus latifolius* was the most active with an EC_{50} value of 5.13 μg/ml (Table 5).

3.3. Tyrosinase inhibition

The oxidation of L-DOPA catalysed by mushroom tyrosinase was inhibited by the plant extracts in a dose-dependent inhibitory effect. The enzyme activity was not suppressed but rather rapidly decreased. At concentrations between 0.14 and 0.47 mg/ml of plant extracts, a 50% inhibitory activity of mushroom tyrosinase was observed (Table 6). The most active extract was from the stem of *Podocarpus elongatus* with an EC_{50} value of 0.14 mg/ml. There was a significant difference at $P = 0.05$ in terms of inhibitory activity between the extracts (Table 6).

3.4. Phenolic analysis

The highest concentration of phenolics was detected in leaves of *Podocarpus elongatus* and *Podocarpus henkelii* (6.94 mg GAE/g and 6.85 mg GAE/g, respectively) (Table 7). Extracts with high phenolic content did not always have a higher condensed tannin, gallotannin or flavonoid content, as was evident with leaf extract of *Podocarpus elongatus* (TP = 6.94 mg GAE/g; CT = 2.47% g/DW; GT = 5.40 μg GAE/g DW; TF = 44.45 μg catechin/g DW) and *Podocarpus henkelii* (TP = 6.85 mg GAE/g; CT = 3.07% g/DW; GT = 5.29 μg GAE/g DW; TF = 52.74 μg catechin/g DW).

Table 5
EC_{50} (μg/ml) for COX-1 and COX-2 inhibition of different plant extracts of *Podocarpus* species.

Podocarpus species	Plant parts	Extraction solvent					
		Petroleum ether		Dichloromethane		80% Ethanol	
		COX-1	COX-2	COX-1	COX-2	COX-1	COX2
Podocarpus elongatus	Leaves	14.22 ± 0.11^{abc}	13.33 ± 0.07^{e}	13.51 ± 0.17^{abc}	14.54 ± 0.17^{f}	21.48 ± 0.15^{abcd}	46.20 ± 0.09^{o}
	Stem	5.02 ± 0.02^{a}	6.06 ± 0.02^{b}	14.95 ± 0.15^{abc}	16.74 ± 0.02^{h}	22.80 ± 0.10^{abcd}	50.79 ± 0.25^{r}
Podocarpus falcatus	Leaves	13.27 ± 0.14^{abc}	19.37 ± 0.11^{i}	11.90 ± 0.28^{abc}	30.74 ± 0.07^{m}	32.98 ± 0.02^{cde}	49.23 ± 0.07^{q}
	Stem	14.55 ± 0.28^{abc}	25.93 ± 0.30^{k}	14.33 ± 0.11^{abc}	30.87 ± 0.02^{m}	31.98 ± 0.03^{bcde}	51.45 ± 0.14^{s}
Podocarpus henkelii	Leaves	13.84 ± 0.33^{cf}	16.95 ± 0.14^{h}	8.83 ± 0.11^{ab}	27.62 ± 0.20^{l}	54.86 ± 0.02^{f}	47.63 ± 0.21^{p}
	Stem	16.21 ± 0.12^{abc}	14.40 ± 0.13^{f}	9.63 ± 0.08^{ab}	12.77 ± 0.2^{d}	54.82 ± 0.67^{f}	54.21 ± 0.06^{t}
Podocarpus latifolius	Leaves	14.33 ± 0.17^{abc}	5.13 ± 0.03^{a}	14.60 ± 0.19^{abc}	24.38 ± 0.12^{j}	41.51 ± 0.17^{def}	57.96 ± 0.31^{u}
	Stem	9.08 ± 0.05^{ab}	9.59 ± 0.18^{c}	9.93 ± 0.01^{abc}	15.80 ± 0.23^{g}	41.02 ± 0.06^{def}	60.09 ± 0.03^{v}

Values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.

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Table 6
Percentage tyrosinase inhibition of leaf and stem extracts of four species of *Podocarpus* used in traditional medicine in South Africa.

Podocarpus species	Plant part	Concentration of methanolic extract (mg/ml)						
		0.031	0.063	0.125	0.250	0.500	1.000	EC_{50}
Podocarpus elongatus	Leaves	18.71	25.90	34.79	45.03	48.62	59.17	0.47 ± 0.001^{d}
	Stem	26.55	40.98	51.83	60.23	64.03	74.03	0.14 ± 0.001^{a}
Podocarpus falcatus	Leaves	30.33	35.90	40.02	47.04	55.15	64.19	0.29 ± 0.002^{b}
	Stem	23.67	29.34	41.19	46.93	50.00	64.19	0.35 ± 0.002^{c}
Podocarpus henkelii	Leaves	18.91	22.25	34.45	38.32	52.01	71.10	0.37 ± 0.003^{g}
	Stem	23.64	25.92	35.72	41.79	50.90	65.22	0.40 ± 0.003^{e}
Podocarpus latifolius	Leaves	6.38	18.32	27.10	36.60	53.75	70.12	0.41 ± 0.005^{h}
	Stem	18.61	27.65	33.56	41.32	54.76	67.25	0.36 ± 0.001^{f}

EC_{50} for positive control: catechin (0.05 mg/ml); values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.

4. Discussion

The extracts from the four *Podocarpus* species investigated showed pronounced antioxidant, anti-inflammatory and tyrosinase inhibitory activities. A number of bioactive compounds such as diterpenes and biflavonoids reported from this genus could be responsible for the observed activities. Amentoflavone, nor- and bis-norditerpenes are taxonomic markers of the genus *Podocarpus* (Ito and Kodama, 1976; Roy et al., 1987) and have accounted for a number of biological activities including anti-inflammatory, antioxidant and tyrosinase inhibitory activities. Biflavonoids such as amentoflavone from this genus inhibit phospholipase A_2 and COX-2 activity, and also regulate pro-inflammatory gene expression both *in vitro* and *in vivo* (Kim et al., 2008). Amentoflavone, was revealed as a potent COX-1 inhibitor ($IC_{50} = 3\ \mu M$) compared to Indomethacin ($IC_{50} = 1\ \mu M$). According to Polya (2003), amentoflavone is a potent cyclooxygenase inhibitor. Diterpenoids such as totarol, totaradiol, 19-hydroxytotarol, 4-beta-carboxy-19-nortotarol and sugiol isolated from *Podocarpus nagi* (Thunb.) Pilg. exhibited a potent antioxidant activity by inhibiting microsomal and mitochondrial lipid peroxidation (Haraguchi et al., 1997). Ferruginol, a terpenoid isolated from *Podocarpus ferrugineus* D. Don has shown anti-inflammatory, antioxidant and gastroprotective properties. The gastroprotective activity contributes to the recovery of the ulcerated lesions and is very important since most anti-inflammatory agents that are active against COX-1 cause gastric mucosal damage (Rodríguez et al., 2006). Amentoflavone and its derivatives showed potent tyrosinase activity against free radical and melanin synthesis in human epidermal melanocytes (Cheng et al., 2007). 2,3-Dihydro-4′,4‴di-*O*-methylamentoflavone is thought to affect cytotoxicity of melanogenic intermediated in melanocytes reducing pigment production (Cheng et al., 2007; Abdillahi et al., 2010). Epicatechin, a polyphenolic, which occurs in the family Podocarpaceae, is known to be an effective inhibitor of tyrosinase activity and inflammation (Kubo et al., 2003; Polya, 2003). Podocarpaceae is one of the non-leguminous families that produce isoflavonoids (Lapčík, 2007). A number of natural and synthetic isoflavonoids are known to possess potent antioxidant and anti-inflammatory activities (Lee et al., 1994; Rahman et al., 2003; Pan et al., 2005).

Plant extracts are composed of a variety of chemical compounds with different pharmacological activities. There is a possibility that these compounds act in a synergistic manner resulting in an overall pharmacological effect. On this note, it is advisable to screen medicinal plant extracts against a number of different *in vitro* assays, avoiding the possibility of losing other potential bioactive compounds when investigating a single biological activity (Houghton et al., 2007). Although antioxidant activities are known to occur in all plants, it was essential to determine if it is found in these species, since reactive oxygen species (ROS) play an important role in the pathogenesis of inflammatory disorders (Kris-Etherton et al., 2004) and the neutralization of free radicals by antioxidants and radical scavengers eases inflammation (Geronikaki and Gavalas, 2006). Other than biflavonoids and diterpenes present in these species, the antioxidant activities displayed may also play a role in the overall anti-inflammatory and anti-tyrosinase effects observed. Melanogenesis is activated by oxidation related processes such as UV radiation among other factors. Melanogenesis requires tyrosinase activity and reactive species such as reactive oxygen and nitrogen species cause oxidative stress to the skin resulting in skin pigmentation and ageing (Cals-Grierson and Ormerod, 2004). Hence, controlling oxidative stress is important for the regulation of melanogenesis, since the antioxidative property of any biological reductant such as reduced glutathione may be closely related to anti-melanogenic actions and regulation of melanin synthesis (Imokawa, 1989). This was recently confirmed by No et al. (2006) and Kim (2007), where the anti-melanogenic activity of both 4,4′-dihydroxybiphenyl and gallic acid were attributed to their antioxidative actions.

The antioxidant activities of flavonoids are attributed to three structural features. These are the *ortho*-dihydroxy group in the B-ring, the conjugation of the B-ring to the 4-oxo group via C2–C3 double bond and the 3- and 5-OH groups with the 4-oxo group (Bors et al., 1990). These structural features have been identi-

Table 7
Total phenolic content, condensed tannins, gallotannin and total flavonoid content of four species of *Podocarpus* used in traditional medicine in South Africa.

Podocarpus species	Plant part	Total phenolics (mg GAE/g DW)[a]	Condensed tannins (% Leuco/g DW)[a]	Gallotannins (μg GAE/g DW)[a]	Total flavonoids (μg catechin/g DW)[a]
Podocarpus elongatus	Leaves	6.94 ± 0.11^{a}	2.47 ± 0.03^{d}	5.40 ± 0.11^{ab}	44.45 ± 4.45^{bc}
	Stem	5.79 ± 0.19^{c}	2.83 ± 0.16^{c}	5.26 ± 0.16^{ab}	41.36 ± 0.30^{cd}
Podocarpus falcatus	Leaves	6.27 ± 0.07^{b}	2.71 ± 0.03^{c}	5.29 ± 0.05^{ab}	37.55 ± 3.89^{d}
	Stem	6.38 ± 0.14^{b}	3.25 ± 0.08^{a}	5.27 ± 0.15^{ab}	49.76 ± 0.77^{ab}
Podocarpus henkelii	Leaves	6.85 ± 0.13^{a}	3.07 ± 0.03^{b}	5.29 ± 0.11^{ab}	52.74 ± 1.06^{a}
	Stem	2.38 ± 0.21^{f}	1.23 ± 0.03^{e}	5.21 ± 0.04^{abc}	13.17 ± 0.55^{f}
Podocarpus latifolius	Leaves	5.38 ± 0.13^{a}	2.78 ± 0.03^{c}	5.05 ± 0.15^{bc}	38.02 ± 5.39^{d}
	Stem	3.86 ± 0.08^{e}	2.55 ± 0.12^{d}	4.97 ± 0.11^{c}	27.18 ± 4.52^{e}

Values are the mean ± standard deviation ($n = 3$); means in the same column followed by different letter(s) are significantly different at $P = 0.05$.
[a] Dry weight basis of the original sample plant parts.

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fied in a number of flavonoids isolated from several species of *Podocarpus* (Markham et al., 1985; Kuo et al., 2008). Flavonoids and flavones derivatives are known to modulate pro-inflammatory gene expression such as inducible nitric oxide synthesis and COX-2. Such compounds have been identified in species of *Podocarpus* and presence of general flavonoids in these species could also be involved in the anti-inflammatory activities observed.

There was no significant correlation between the antioxidant activity exhibited in the present assays and total phenolic content. The correlation coefficient (r^2) in all the plant extracts was less than 0.1 ($r^2 \leq 0.2$). It has been reported that different classes of phenolics have varying antioxidative strength and that synergism of polyphenolics, (in our case condensed tannins, gallotannins and flavonoids) with one another or with other components present in an extract may contribute to the overall observed antioxidant activity (Shahidi et al., 1994). Various naturally occurring anti-melanogenic reagents contain a phenolic structure (Lim, 1999; No et al., 2004; Kim, 2007). Many tyrosinase inhibitors are polyphenol derivatives of flavonoids (Briganti et al., 2003; Seo et al., 2003), thus the presence of flavonoid in these species and flavonoids derivatives in this genus may contribute to the anti-tyrosinase activity observed.

Inhibition of COX-1 may lead to adverse effects such as gastric ulceration and increase risk of adverse cardiovascular events. As a result much emphasis has been placed on selective COX-2 inhibitors. However, the constitutive expression of COX-2 in some tissues has aroused concerns about the side effects of inhibiting this isoenzyme. COX-2 inhibitors increase the risk of artherothrombosis and are associated with a moderate increased risk of vascular events (Kearney et al., 2006). The undesirable effects of COX inhibitors may be attributed to the 5-lipoxygenase pathway which is up-regulated during COX blockade. Hence, inhibition of both COX and 5-LOX may constitute a valuable alternative to NSAIDs and selective COX-2 inhibitors. Since *Podocarpus* species showed inhibition activity against both COX-1 and COX-2, the use of these plants as traditional anti-inflammation agents should be carefully monitored, especially in terms of dosage.

The present findings support the use of the four *Podocarpus* species in traditional medicine and indicate their potential in providing biological active compounds. Since the activities exhibited by these species were from leaves and stems and not the bark that is generally used, substitution of the bark with these plant parts will impact on conservation. This will add more value on the sustainable uses of these species, especially in areas where they are protected due to overexploitation. The data will also add to the knowledge base needed to advance the local management of disease.

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