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Full Length Research Paper

In vitro 5-Lipoxygenase inhibition of polyphenolic antioxidants from undomesticated plants of South Africa

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Aqueous and methanolic extracts of 18 leafy vegetables from South Africa were investigated for their free radical scavenging activity, total phenolic content and anti-inflammatory properties. *Amaranthus dubius, Amaranthus hybridus, Amaranthus spinosus, Asystasia gangetica, Bidens pilosa, Centella asiatica, Ceratotheca triloba, Chenopodium album, Cleome monophylla, Emex australis, Galinsoga parviflora, Justicia flava, Momordica balsamina, Oxygonum sinuatum, Physalis viscosa, Portulaca oleracea, Senna occidentalis and Solanum nigrum* were used in this study. In the radical scavenging studies using 2,2-diphenyl-2-picrylhydrazyl hydrate(DPPH), methanolic extracts from six plants almost completely inhibited DPPH absorption (*J. flava*-96.6%, *P. oleracea*-96.5%, *M. balsamina*-94.7%, *O. sinuatum*-92.1%, *S. nigrum*-92.0% and *A. hybridus*-90.5%), whereas methanolic extracts from *E. australis*-78.6% and *G. parviflora*-76.5% showed lowest activities among the plants studied. Aqueous extracts were considerably less effective radical scavengers compared to methanolic extracts showing highest activity with *J. flava*-48.0% and lowest with *A. gangetica*-36.2%. A correlation between radical scavenging activity of extracts with total phenolic content was observed. Out of 18 plants studied for anti-inflammatory activity, methanolic extracts from eight plants showed significant inhibition of 5-lipoxygenase (5-Lox) activity. Among the plants studied *Bidens pilosa* (IC_{50} 21.8 µg/ml) showed maximum anti-inflammatory activity while *Emex australis* (IC_{50} 81.4 µg/ml) showed minimum inhibition of 5-Lox activity. Large amounts of phenolic compounds may contribute towards the antioxidant and anti-inflammatory properties among the plants studied.

Key words: Leafy vegetables, radical scavenging, DPPH, total phenolics, 5-lipoxygenase, anti-inflammatory.

INTRODUCTION

The uncontrolled production of oxygen free radicals and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. The antioxidants when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Percival, 1998; Young and Woodside, 2001; Atoui et al., 2005).

The physiological role of free radical and hydroxyl free radical-scavengers is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. The key role for free radicals is as major contributors to ageing and to degenerative diseases of ageing, such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction (Ames et al., 1990; Percival, 1998; Young and Woodside, 2001). Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants (Percival, 1998).

When the availability of antioxidants is limited, this damage can become cumulative and debilitating oxidetive stress results. Antioxidants are capable of stabilizing, or deactivating free radicals before the latter attack cells and biological targets. They are therefore critical for maintaining optimal cellular and systemic health and well-being (Percival, 1998). Most antioxidants isolated from higher plants are polyphenols, which show biological activity as antibacterial, anti-carcinogenic, anti-inflammatory, anti-viral, anti-allergic, estrogenic and immune-stimulating

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Ing effects (Larson, 1988).

The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential. Synthetic anti-oxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ) have been used widely as antioxidants in foods, but concerns over the safety of use have led towards interests in natural anti-oxidants (Wanasundara and Shahidi, 1998). These synthetic antioxidants are substituted phenolic compounds, and subsequently much of the research on natural anti-oxidants has also focused on phenolic compounds, in particular the flavonoids and hydroxycinnamic acids (Martı´nez-Valverde et al., 2002). Phenolic compounds also possess an array of potentially beneficial lipoxygenase inhibitory and anti-oxidant properties; they have been used for the treatment of inflammatory diseases (Sreejayan and Rao, 1996).

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role. Prostaglandins are involved in the complex process of inflammation and are responsible for the pain (Jager et al., 1996). It can be metabolized by the cyclooxygenase (COX) pathway to prostaglandins and thromboxane A2, or by the lipoxygenase (LOX) pathway to hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's), which are important biologically active mediators in a variety of inflammatory events (Alitonou et al., 2006, Piper et al., 1994). Upon appropriate stimulation of neutrophils, arachidonic acid is cleaved from membrane phospholipids and can be converted to leukotrienes and prostaglandins through 5-lipoxygenase(5-Lox) or cyclooxygenase(Cox) pathways respectively (Bouriche et al., 2005). Furthermore, inflammatory processes also involve reactive oxygen species started by leucocyte activation. Grabmann et al. (2000) found that eucalyptus and myrtle essential oils attenuated leucocyte activation by scavenging hydroxyl radicals indirectly produced by leucocyte degranulation, thereby interfering with inflammatory processes by acting as antioxidants. Therefore, screening of antioxidant properties may provide important information about the potential activity of a drug on inflammatory processes (Njenga and Viljoen, 2006) The establishment of new *in vitro* test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. The plant lipoxygenase pathway is in many respects the equivalent of the 'arachidonic acid cascades' in animals (Gardner, 1991). For this reason, the *in vitro* inhibition of soybean lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential (Abad et al., 1995). A combination of this assay with an evaluation of the radical scavenging activity by the 1,1-diphenyl-1-picrylhydrazyl (DPPH) method (Choi, 2002) constitutes a good indication on the potential anti-inflammatory activity of a drug (Alitonou et al., 2006).

Many of the plant anti-oxidants are polyphenols which have carbon-based aromatic phenyl-ring compounds which are easily oxidized to quinones by reactive oxygen species, a property that helps account for their free radical scavenging capacity. The leaves of many traditional leafy vegetables supplement the diets, which consist mainly of cereals and grains (Van Wyk and Gericke, 2000). Most of these plants lack comprehensive data that can corroborate the traditional knowledge. The aim of this work was to investigate 18 traditional leafy vegetables found in the Kwa-Zulu Natal province of South Africa for their anti-oxidative and anti-inflammatory potential, and correlate this to total phenolic compounds.

MATERIALS AND METHODS

Plant material

Eighteen traditional leafy vegetables were used in this study. They were collected from different locations in Kwa-Zulu Natal, South Africa. Table 1 lists the leafy vegetables and consumption practices used in this study with the following information: scientific name, family, English and Zulu names. Local floristic keys were used for determining the species. Data on different species and the Zulu names have been sourced from publications on the flora of South Africa (Odhav et al., 2007; Hutchings, 1996). Voucher specimens are housed in the Ward Herbarium, University of Kwa-Zulu-Natal, Westville campus, Durban, South Africa.

Sample extraction

The leaves (100 g) were dried at 30ºC in a oven (Memmert) and powdered in a Salton blender. Ten grams were dissolved in 200 ml of dd H_2O/ 80% methanol (vol/vol) for one hour and filtered with a Whatman No. 1 filter paper. The filtrate was then concentrated to 15 ml using a Buchi RE 121 Roto-evaporator including a Buchi 461 water bath set at a temperature of 50ºC. This concentrate was freeze dried using a Virtis Benchtop Freeze drier.

DPPH radical scavenging assay

The radical scavenging activity of plant extracts was measured using the stable free radical scavenger, DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate), decoloration assay described by Choi (2002). The freeze dried plant samples (1.0 mg/ml) from each of the plant extracts were diluted in ethanol to give final concentrations of 500, 250, 100, 50 and 10 µg/ ml. One ml of a 0.3 mM DPPH in ethanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature for 30 min. The resulting colour was measured at A518 spectrophotometrically (Varion Cary I-E UV visible spectrophotometer). The radical scavenging activity was measured as the decolourization percentage of the test sample using the following formula:

DPPH Scavenging capacity % =100 - [(Absorbance of sample - Absorbance of blank) x 100/Absorbance of control]

While ethanol (1.0 ml) plus plant extract solution (2.5 ml) was used as a blank, DPPH solution plus ethanol was used as a negative control. The positive controls were DPPH solution plus each 1 mM Rutin.

Table 1. List of species and consumption practices of leafy vegetables in Kwa-Zulu Natal area of South Africa.

Family	Scientific name	English Name	Zulu Name	Consumption
Acanthaceae	*Asystasia gangetica*	hunter's spinach	isihobo	during famine
Acanthaceae	*Justicia flava*	yellow justicia	ipela	occasionally
Amaranthaceae	*Amaranthus dubius*	wild spinach	imbuya	regularly
Amaranthaceae	*Amaranthus hybridus*	cockscomb	imbuya	regularly
Amaranthaceae	*Amaranthus spinosus*	spiny pigweed	imbuya	regularly
Apiaceae	*Centella asiatica*	marsh pennywort	icudwane	during famine
Asteraceae	*Bidens pilosa*	black jack	amalenjane	regularly
Asteraceae	*Galinsoga parviflora*	gallant soldier	ushukeyana	regularly
Capparaceae	*Cleome monophylla*	spindle-pod	isiwisa	regularly
Chenopodiaceae	*Chenopodium album*	fat hen	imbikilicane	regularly
Cucurbitaceae	*Momordica balsamina*	balsam apple	umkaka	regularly
Fabaceae	*Senna occidentalis*	coffee senna	isinyembane	during famine
Pedaliaceae	*Ceratotheca triloba*	wild foxglove	udonqabathwa	regularly
Polygonaceae	*Emex australis*	devil's thorn	inkunzane	occasionally
Polygonaceae	*Oxygonum sinuatum*	starstalk	untabane	during famine
Portulacaceae	*Portulaca oleracea*	purslane	madilika	regularly
Solanaceae	*Physalis viscosa*	sticky gooseberry	uqadolo	during famine
Solanaceae	*Solanum nigrum*	black nightshade	umsobosobo	regularly

Estimation of total phenolic compounds in plant extracts

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu method (Miliauskas et al., 2004). For the preparation of calibration curve 1 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml ethanolic gallic acid solutions were mixed with 5 ml Folin–Ciocalteu reagent (diluted three-fold) and 4 ml (75 g/l) sodium carbonate. The absorption was read after 30 min at 200ºC at 765 nm and the calibration curve was drawn. One ml methanolic plant extract (10 g/l) was mixed with the same reagents as described above, and after 1 h the absorption was measured. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$C = c.V/m$

Where: C = total content of phenolic compounds (mg/g) plant extract in GAE; c = the concentration of gallic acid established from the calibration curve (mg/ml); V = the volume of extract in ml; and m = the weight of pure plant methanolic extract in g.

Determination of the 5- lipoxygenase activity

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13- hydroperoxy linoleic acid was followed spectro-photometrically by the appearance of a conjugate diene at 234 nm on a UV/visible spectrophotometer (Varion Cary 1E UV- Visible spectrophotometer). Nordihydroguaiaretic acid (NDGA) and Rutin known inhibitors of soybean lipoxygenase, were used as controls. The reaction was initiated by the addition of aliquots (50 µl) of a soybean lipoxygenase solution (prepared daily in potassium phosphate buffer M pH 9.0) in a sufficient concentration to give an easily measurable initial rate of reaction to 2.0 ml of sodium linoleate (100 µM) in phosphate buffer; the enzymatic reactions were performed in absence or in presence of inhibitor and their kinetics were compared. The inhibitors were dissolved in DMSO in such a manner that an aliquot of each (30 µl) yielded a final concentration of maximum 100 ppm in each assay. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 30 µl of phosphate buffer (pH 9.0) instead of 30 µl of the inhibitor solution). Each inhibitor concentration was tested in triplicate and the results averaged; the concentration that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration (Njenga and Viljoen, 2006; Baylac, 2003). Aqueous extracts (IC_{50} ≥100 µg/ml) were not taken in this study. A negative result in the lipoxygenase assay does not necessarily mean that the plant is without anti-inflammatory activity. The active compounds could work at other sites in the complex process of inflammation (Jager et al., 1996).

Statistical analysis

All the analysis were carried out in triplicate and the results were expressed as the mean ±SD. Correlation co-efficient (R) to determine the relationship between two or more variables among Radical Scavenging activity tests and content of total phenolic compounds were calculated using MS Excel software (CORREL statistical function). Regression analysis was used to calculate IC_{50}, defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction.

RESULTS AND DISCUSSION

For all the plants the methanolic extracts were more effective DPPH radical scavengers than the aqueous extracts (Table 2). Almost complete DPPH absorption was found with methanolic extracts from *Justicia flava* (96.6%), *Portulaca oleracea* (96.5%), *Momordica balsamina* (94.7%), *Oxygonum sinuatum* (92.1%), *Solanum nigrum* (92.0%) and *Amaranthus hybridus* (90.5%).

Table 2. Antioxidant values of the plant extracts among leafy vegetables from Kwa-Zulu Natal, South Africa.

Plant name	Percent scavenging activity of methanolic extract (a, b)	Percent scavenging activity of aqueous extract(a, b)
Amaranthus dubius	78.4±0.32	42.6±0.14
Oxygonum sinuatum	92.1±0.26	44.1±0.25
Asystasia gangetica	84.7±0.38	36.2±0.18
Galinsoga parviflora	76.5±0.44	42.9±0.22
Physalis viscosa	82.4±0.30	40.7±0.12
Justicia flava	96.6±0.54	48.0±0.08
Amaranthus spinosus	88.2±0.22	40.6±0.42
Solanum nigrum	92.0±0.18	42.1±0.36
Amaranthus hybridus	90.5±0.24	42.0±0.12
Momordica balsamina	94.7±0.36	42.5±0.32
Centella asiatica	88.2±0.12	39.0±0.24
Chenopodium album	82.5±0.38	40.8±0.18
Emex australis	78.6±0.22	39.2±0.26
Senna occidentalis	82.4±0.26	40.3±0.14
Ceratotheca triloba	84.9±0.16	36.7±0.28
Bidens pilosa	88.7±0.34	40.5±0.38
Cleome monophylla	84.7±0.26	36.4±0.26
Portulaca oleracea	96.5±0.38	44.3±0.08

[a]Mean values obtained from experiments performed in triplicate.
[b]Mean value determined graphically and standard deviation.

Others such as *Emex australis* (78.6%) and *Galinsoga parviflora* (76.5%) had lower activities. The aqueous extracts were considerably less effective radical scavengers showing highest activity with *J. flava* (48.0%) and lowest with *Asystasia gangetica* (36.2%).

The content of phenolic compounds (mg/g) in methanolic extracts (Table 3) expressed in GAE, varied between 21.1 and 42.4. The highest amounts were found in the extracts of *A. hybridus* (42.4) followed by *J. flava* (40.6). Furthermore, plants showing high radical scavenging activity [*J. flava* (96.65%) and *M. balsamina* (94.7%)] also contain high amounts of phenolic compounds (40.6 and 39.8 respectively). A positive correlation (R^2 = 0.78) was found, suggesting that the antioxidant capacity of the methanolic plant extracts is due to a great extent to the polyphenol content. The quantitative correlation between the antioxidant activity and the content of polyphenols in most of the plants (Figure 1) indicates that phenolic compounds are likely to contribute to the radical scavenging activity among these plant extracts.

Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation (Hotta et al., 2002) and studies on antioxidant and anti-inflammatory activities of methanolic extracts from *Ledum groenlandicum* (Dufour, 2007) have indicated a possible correlation between the antioxidant activity and the phenolic compounds. Out of 18 plants studied for anti-inflammatory activity, methanolic extracts from eight plants showed significant inhibition of 5-lipoxygenase (5-Lox) activity (Table 4). Among the plants studied *Bidens pilosa* (IC_{50} 21.8 µg/ml) showed maximum anti-inflammatory activity while *Emex australis* (IC_{50} 81.4 µg/ml) showed minimum inhibition of 5-Lox activity. An interesting finding from this study shows that the members of family Asteraceae have high levels of anti-inflammatory activity [(*Bidens pilosa* (IC_{50} 21.8 µg/ml), *Galinsoga parviflora* (IC_{50} 30.7 µg/ml) whereas members from family Solanaceae

S. nigrum (IC_{50} 27.1 µg/ml), *P. viscosa* (IC_{50} 29.5 µg/ml)] show moderate activity and those from *Amaranthaceae* like *A. hybridus* (IC_{50} 77.2 µg/ml), *A. dubious* (IC_{50} 69.4 µg/ml) and *A. spinosus* (IC_{50} 57.3 µg/ml) showed minimum inhibition of 5-Lox activity. Earlier studies (Njenga and Viljoen, 2006; Abajo et al., 2004; Matu and Van Staden, 2003) found similar results with some members of the *Asteraceae* family, whereas *O. sinuatum* (IC_{50} 32.1 µg/ml), *E. australis* (IC_{50} 81.4 µg/ml) from Polygonaceae and *J. flava* (IC_{50} 31.4 µg/ml), *A. gangetica* (IC_{50} 55.0 µg/ml) from Acanthaceae showed marked variance in 5-Lox inhibition.

Antioxidants are known to inhibit plant lipoxygenases (Ammon et al., 1993). Studies have implicated the oxygen free radicals in the process of inflammation and phenolic compounds may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity, and may serve as a scavenger of reactive free radicals which are produced during arachidonic acid me-

Table 3. Amount of total phenolic compounds (mg/g) in gallic acid equivalents among leafy vegetables from Kwa-Zulu Natal, South Africa

Plant name	Total phenolic compounds, (mg/g) in methanolic extract (a,b)	Total phenolic compounds, (mg/g) in aqueous extract (a.b)
Amaranthus dubius	26.2±0.34	18.1±0.24
Oxygonum sinuatum	39.4±0.14	24.6±0.18
Asystasia gangetica	28.3±0.26	16.5±0.22
Galinsoga parviflora	24.1±0.30	15.3±0.36
Physalis viscosa	32.0±0.41	19.6±0.30
Justicia flava	40.6±0.38	26.2±0.16
Amaranthus spinosus	32.3±0.14	21.8±0.41
Solanum nigrum	37.6±0.22	16.4±0.31
Amaranthus hybridus	39.6±0.32	28.4±0.08
Momordica balsamina	39.8±0.54	25.2±0.44
Centella asiatica	31.8±0.38	16.1±0.28
Chenopodium album	26.8±0.12	15.4±0.22
Emex australis	27.4±0.36	20.8±0.18
Senna occidentalis	27.6±0.12	21.6±0.42
Ceratotheca triloba	35.2±0.44	14.6±0.24
Bidens pilosa	38.1±0.08	24.4±0.32
Cleome monophylla	32.6±0.20	12.5±0.18
Portulaca oleracea	37.6±0.14	23.6±0.20

[a]Mean values obtained from experiments performed in triplicate.
[b]Mean value determined graphically and standard deviation.

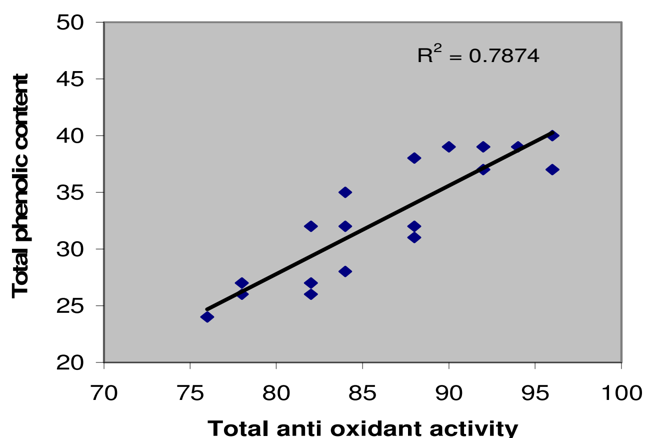

Figure 1. Correlation between total anti-oxidant activity and total polyphenol content of methanolic extracts of 18 leafy vegetables from Kwa-Zulu Natal, South Africa.

metabolism (Trouillas et al., 2003; Sreejayan and Rao, 1996). The anti-inflammatory activities of the eighteen plant extracts could be explained by the potent inhibitory effects of their phenolic compounds on arachidonic acid metabolism through the lipoxygenase pathway. It is possible that the correlation observed between the three tests is due to the considerably high amounts of phenolic compounds among the plants studied.

Table 4. Anti-inflammatory activity (5-Lipoxygenase inhibition) of methanolic extracts among leafy vegetables from Kwa-Zulu Natal, South Africa.

Plant name	5-Lox IC_{50} (µg/ml)*
Amaranthus dubius	69.4
Oxygonum sinuatum	32.1
Asystasia gangetica	55.0
Galinsoga parviflora	30.7
Physalis viscosa	29.5
Justicia flava	31.4
Amaranthus spinosus	57.3
Solanum nigrum	27.1
Amaranthus hybridus	77.2
Momordica balsamina	40.3
Centella asiatica	38.6
Chenopodium album	33.2
Emex australis	81.4
Senna occidentalis	43.5
Ceratotheca triloba	56.1
Bidens pilosa	21.8
Cleome monophylla	32.0
Portulaca oleracea	37.9
NDGA	4.1
Rutin	7.3

*Inhibition (%) of 5-lipoxygenase activity obtained and IC_{50} values represent the mean ±S.D. of three determinations.

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