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Full Length Research Paper

Antioxidant activity and cytotoxicity effect of flavonoids isolated from *athrixia phylicoides*

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Bioassay-guided fractionation of an ethanolic extract from aerial parts of *Athrixia phylicoides* using silica and sephadex column chromatography led to the isolation of three flavonoids. The compounds were identified as: 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol (1), 3-0-demethyldigicitrin (2), and Quecertin (3). Isolated compounds together with ethanol crude extract were tested for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH)-spectrophotometric assay, while cytotoxicity effect was determined using XTT colorimetric assay. The crude extract showed a concentration-dependent radical scavenging activity with EC_{50} value of 10.64 ± 0.08 µg/ml. Compound 3 was the most potent radical scavenger, exhibiting EC_{50} value of 1.27 ± 0.25 µg/ml, followed by compound 1 and 2 showing 2.74 ± 0.10 and 3.41 ± 0.09 µg/ml respectively. The crude extract showed no or little toxicity on Vero cells at lower concentrations tested exhibiting the IC_{50} value of 107.8 ± 0.13 µg/ml. Compound 3 showed minimal toxicity effect by exhibiting IC_{50} value of 81.38 ± 0.33 µg/ml as compared to compound 2 (IC_{50}, 28.92 ± 0.12 µg/ml) and compound 1 (IC_{50}, 27.91 ± 0.18 µg/ml). The results obtained from this study provide a clear rationale for the medicinal uses of *A. phylicoides*.

Key words: *Athrixia phylicoides*, antioxidant activity, cytotoxicity, 2,2-diphenyl-1-picrylhydrazyl, flavonoids, vero cells.

INTRODUCTION

Athrixia phylicoides is an aromatic shrub indigenous to South Africa, belonging to the Asteraceae family and grows naturally in the mountainous parts of South Africa, from the Eastern Cape in the south, to the Soutpansberg in the Limpopo province in the north (Van Wyk and Gericke, 2000). It is commonly known as Bush tea (English), Umtshanela (Zulu), Icholocholo (Xhosa), Sephomolo (Sotho), and Luphephetse (Swazi) (Joubert et al., 2008). The dried leaves and fine twigs of this plant have traditionally been used by many South Africans as herbal tea and medicinal decoctions. Plant infusions are used by the Zulus as blood purifier or cleanser, to treat boils, headaches, infested wounds and cuts (Du Toit et al., 2001; Roberts, 1990; Wyk and Gericke, 2000).

The plant is also used as a gargle for infected throats, and roots decoctions serve as purgative and cough remedy (Mashimbye et al., 2006; Mcgaw et al., 2007). The Vhavenda people drink the extracts made from the leaves and roots as an aphrodisiac (Mabogo, 1990). Furthermore, dried stem of bush tea are tied up in bundles for aromatic brooms and traded on a small scale in Limpopo province (Banerjee et al., 2005). The previous phytochemical study of *A. phylicoides* crude extract have resulted in the isolation of new flavonoid, 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol (Mashimbye et al., 2006). It has been reported that the ethanol extract have antioxidant and cytotoxic activities, and no caffeine contents or pyrrolizidine alkaloids were detected (Mcgaw

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et al., 2007).

To our best knowledge, no previous investigation has been done on the biological activities of compounds isolated from this plant. The aim of this study was to investigate the antioxidant activity and cytotoxicity effect of the crude extract and isolated flavonoids from the aerial parts of *A. phylicoides*

MATERIALS AND METHODS

Plant material

The aerial parts of *A. phylicoides* (Asteraceae) were collected from Venda in Limpopo Province in South Africa and allowed to dry under the shade for 48 h. A voucher specimen (E.M.12) was deposited at the H.G.W.J. Schweickerdt herbarium at the University of Pretoria. The dried small cuts of *A. phylicoides* (4 kg) were macerated in ethanol (5 L) for 48 h and filtered with Buchner funnel. The filtrate was then evaporated to dryness under reduced pressure to give a dark green extract (130 g).

Isolation of the antioxidant compounds

One hundred and ten grams (110 g) of crude extract were subjected to column chromatography on silica gel 60 and eluted with a solvent gradient of n-hexane-ethyl acetate mixtures in 100:0 to 0:100 ratios. The column was then washed with ethyl acetate (100%), methanol ethyl acetate (2:8), and 100% methanol. Thirty-four fractions of 1000 ml each were collected and combined on the basis of TLC profile into twelve major fractions (A-L). These twelve fractions were assayed qualitatively for antioxidant activity. Fraction F (46.45 g) contained many antioxidant compounds which was then subjected to silica gel column chromatography eluted with n-hexane-ethyl acetate mixtures of increasing polarity followed by 100% methanol. A total of 20 sub-fractions were obtained. The chromatography of these sub-fractions on sephadex column eluted with 100% methanol yielded flavonoids.

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

Antioxidant activities of the ethanol extract of *A. phylicoides* and isolated compounds were investigated using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging assay previously described by Rangkadilok (Rangkadilok et al., 2007) with slightly modifications. Briefly, 200 µl of distilled water was added to the first top wells and the remaining wells of 96 wells ELISA plates were filled with 110 µl as a medium. Twenty microliters of crude extract/ compounds was added to the first top wells and followed by double dilution. Later, 90 µl of 90 µM DPPH methanolic solution was added to each well. Final concentrations of the ethanol extract of *A. phylicoides* ranged from 3.9 to 500 µg/ml and isolated compounds ranged from 0.8 to 100 µg/ml.

Ascorbic acid (vitamin C) was used as the positive control, ethanol was used as negative control and distilled water as a blank. The plates were covered with aluminium foil and left to stand for an hour at room temperature. The radical scavenging capacities of the extract/compounds were determined by using a BIO–TEK Power Wave Multiwell plate reader (A.D.P., Weltevreden Park, South Africa) at 550 nm to measure the disappearance of DPPH at 550 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. This activity is given as percentage DPPH radical-scavenging that is calculated in the equation:

$$\% \text{ DPPH radical-scavenging} = (AC - AS)/AC \times 100,$$

Where AC is the absorbance of the control solution (containing only DPPH), AS is the absorbance of the sample in DPPH solution. The percentage of DPPH radical-scavenging was plotted against the plant extract/compounds concentrations (µg/ml) to determine the concentration of extract required to scavenge DPPH by 50% (EC_{50}).

Statistical analysis

Each of the measurements described above were carried out in triplicate experiments, and the results are reported as the mean and standard deviation. EC_{50} was estimated by sigmoid non-linear regression using SigmaPlot 2000 Demo (SPSS Inc., Chicago, IL, USA).

Cell culture

The cytotoxicity of the *A. phylicoides* and isolated compounds was tested against Vero cells lines. The cells were cultured and maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillin, 10 µg/ml streptomycin, 0.25 µg/ml fungi zone and 10% foetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. Cells were subcultured in a ratio of 1:3 every second to third day after trypsinization of confluent cultures (American Tissue Culture Collection).

Cytotoxicity assay

The cytotoxicity of crude extract and pure compounds isolated from *A. phylicoides* was investigated by the XTT (sodium 3´-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) colorimetric assay using Cell Proliferation Kit II (Roche Diagnostics GmbH) as previously described by Tshikalange 26. The final concentrations of crude extract in the wells were 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/ml. The final concentrations of pure compounds in the wells were 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 µg/ml. Zelaralenone was used as positive control.

The optical densities were measured at 450 nm (690 nm reference wavelength) with an ELISA plate reader (KC Junior program). The concentration of the extract/compounds at which 50% (IC_{50}) of the Vero cells were alive until the 4th day was considered as to be the highest concentration which is non-toxic to the cells. The IC_{50} values were calculated by graph pad prism 4 programme. The assay was carried out in triplicate.

RESULTS AND DISCUSSION

Isolation of antioxidant compounds

The chemical structures of isolated compounds were identified using Nuclear Magnetic Resonance (NMR) spectra and direct comparison of the spectral data of each isolated compound with the published data. Compound 1 was identified as 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol and has been isolated from the same plant before. Compound 2 was identified as 3-0-demethyldigicitrin and has been previously isolated from

Table 1. Antioxidant activity and cytotoxicity of crude extract and isolated compounds.

Sample	EC_{50} (µg/ml)	IC_{50} (µg/ml)
Extract	10.64 ± 0.08	107.8 ± 0.13
Compound 1	2.74 ± 0.10	27.91 ± 0.18
Compound 2	3.41 ± 0.09	28.92 ± 0.12
Compound 3	1.270 ± 0.25	81.38 ± 0.33
Vitamin C	2.656 ± 0.05	-
Zelaralenone	-	2.6 ± 0.31

Zieridium pseudobtusifolium (Johannes et al., 1994). Compound 3 was identified as quercetin, the widely distributed flavonol. It has been previously isolated from the aerial parts of *Hypericum hyssopifolium* (Cakir et al., 2003), leaves of *Castanea crenata* (Lee et al., 1999), aerial parts of *Epimedium brevicornum*, flowers of *Campsis radicans*, roots of *Aster tataricus*, seeds of *Cuscuta chinensis*, and fruits of *Cornus officinalis* (Cai et al., 2004).

DPPH scavenging assay

The concentration of an antioxidant needed to decrease the initial DPPH concentration by 50% (EC_{50}) was used to measure the antioxidant activity of crude extract and isolated compounds (Du Toit et al., 2001; Sanchez et al., 1998; Tshikalange et al., 2007). The EC_{50} values of crude extract and isolated compounds are reported in Table 1. The crude extract and all the isolated compounds tested showed a potent DPPH radical scavenging activity with EC_{50} values ranging from 1.27 to 10.64 µg/ml. The crude extract showed a concentration-dependent radical scavenging activity with EC_{50} value of 10.64 ± 0.08 µg/ml. The ethanol crude extract from *A. phylicoides* have been previously reported to be a potent free radical scavenger (Eloff, 1998; Mativandlela et al., 2006; Mcgaw et al., 2007; Papiez et al., 2008). Of all compounds isolated, compound 3 was the most potent radical scavenger, exhibiting EC_{50} value of 1.27 ± 0.25 µg/ml, followed by compound 1 (EC_{50}, 2.74 ± 0.10 µg/ml), and Compound 2 (EC_{50}, 3.41 ± 0.09 µg/ml) as the least active compound.

The lower the EC_{50} value the higher is the antioxidant activity (Atoui et al., 2005; Banerjee et al., 2005; Chan et al., 2007; Loo et al., 2008). In our results, it is important to note that Compound 3 (quercetin) showed a higher antioxidant activity compared to standard control (vitamin C). Our results are in line with the study by Loo et al. (2008), which reported that three compounds isolated from *Rhizophora apiculata* had higher scavenging activity than vitamin C. It is well-documented that flavonoids such as quercetin, catechin and kaempferol are potent antioxidants agent 20 times than vitamin C and vitamin E (Chow et al., 2005; Martini et al., 2004). The activity shown by quercetin can be attributed to presence of the 3- and 5-OH groups in A-ring, a catechol moiety of the B-ring and the 2,3-double bond in conjugation with a 4-oxofunction of a carbonyl group in the C-ring (Kumarasamy et al., 2002).

Cytotoxicity effect

The cytotoxic effects (IC_{50}) of crude extract and isolated compounds on Vero cells are reported in Table 1. The crude extract showed no or little toxicity at lower concentrations tested exhibiting the IC_{50} value of 107.8 ± 0.129 µg/ml. Toxicity effects were seen at higher concentration tested (400 µg/ml), with cell viability of less than 40%. High toxicity of ethanol crude extract on Vero cells using MTT cytotoxicity assay have been reported However, the aqueous extracts prepared from the same species have been reported to be not toxic on Vero cells (Macgaw et al., 2007), and Wistar rat model following sub-chronic ingestion (Chellan et al., 2008; Mathibe et al., 2008). To our best knowledge, there are no toxic reports of traditionally prepared (aqueous) *A. phylicoides* since it has been discovered many years as a beverage. Compound 3 showed minimal toxicity effect by exhibiting IC_{50} value of 81.38 ± 0.33 µg/ml as compared to compound 2 (IC_{50}, 28.92 ± 0.12 µg/ml) and Compound 1 (IC_{50}, 27.91 ± 0.18 µg/ml). It is well documented that Compound 4 is a potent antitumor agent (Chow et al., 2005). The toxicity exhibited by Compound 2 on Vero cells is in consistence with the findings by Johannes et al., 1994 which showed a high toxicity against carcinoma cells.

Conclusion

Crude extract and Compound 3 showed good antioxidant activity and less cytotoxicity. There is a growing interest in the investigation of natural antioxidant compounds from plants, since they contain secondary metabolites with structural diversity. In comparison with the synthetic compounds that are currently available, a good natural antioxidant will have a higher potency and lower toxicity. In this study compounds isolated (1, 2 and 3) from *A.*

phylicoides revealed a strong dose response antioxidant activity, but Compound 1 and 2 also showed some cytotoxicity on Vero cell lines. From these results obtained in this study, it is clear that more research is needed to validate the toxicity of the isolated compounds on other cell lines and further investigation of other secondary metabolites from *A. phylicoides*. These in vitro activities obtained from this study provide a clear rationale for the medicinal uses of *A. phylicoides* by South Africans for making herbal tea in the treatment of many ailments.

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