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Antioxidant and Anti-Proliferative Capacity of a Dichloromethane Extract of *Dicerocaryum senecioides* Leaves

Leseilane J. Mampuru[1,*], Pirwana K. Chokoe[1], Maphuti C. Madiga[1], Annette Theron[2], Ronald Anderson[2] and Matlou P. Mokgotho[1]
[1]*Department of Biochemistry, Microbiology and Biotechnology, Faculty of Science and Agriculture, University of Limpopo, Sovenga,*
[2]*Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria and Tshwane Academic Division of the NHLS, Pretoria, South Africa*

1. Introduction

Plant derivatives have been used over the years to treat a wide variety of ailments, from microbial infections to various forms of neoplastic growth. The isolation and characterisation of novel compounds that might serve as leads for the development of new and effective drugs from medicinal plants has now become an area of much interest worldwide (de las Heras et al., 1998; de Mesquita et al., 2009, 2011; Russo et al., 2010; Suffness & Pezzuto, 1990; Taylor et al., 2001). To this end, plants have been shown to have very high anti-oxidative activity that makes them potential anti-proliferative, anti-invasive and pro-apoptotic agents. Indeed, in drug discovery or drug assessment using cell lines, researchers endeavour to find compounds that lead to the triggering of apoptosis or programmed cell death in diseased cells such as cancer or HIV infected cells (Cragg et al., 1993; Cragg & Newman, 2000; de Mesquita et al., 2011; Huerta-Reyes et al., 2004; Klos et al., 2009). A candidate drug is, therefore, introduced to the cells and its effects ascertained. The most ideal is a compound that is potent at low concentrations and discriminates between diseased and normal cells (Cochrane et al., 2008; Wang, 1998).

The chemopreventive effects of vegetables and fruits are attributed to a combined effect of various phenolic phytochemicals which are generally antioxidant in nature, along with vitamins, dietary fibers, sulforophanes (in broccoli), selenium, carotenes, lycopenes, indoles, and isoflavones (Gurib-Fakim, 2006). These polyphenolic compounds possess known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, as well as anti-proliferative and anti-inflammatory actions, anti-bacterial, and anti-viral activities to some extent (Frankel, 1995; Pinmai et al., 2008). Subsequently, the intake of herbal remedies and some common dietary supplements rich in antioxidants and micronutrients has been associated with reduced risks of cancer, diabetes and other degenerative disorders associated with inflammation and ageing (Bandera et al., 2007; Liu et

al, 2008; de Mesquita et al., 2009; Parsons et al., 2008). Despite the many concerns regarding the degree of bioavailability and biotransformation of these phytochemicals in *in vivo* experimental settings, as compared to the *in vitro* situations, this wide group of natural molecules nonetheless represents a promising class as anticancer drugs, due to their multiple targets in cancer cells and their limited toxic effect on normal cells (Manach et al., 2005). In fact, many of the plant-derived phytochemicals have already been isolated, characterised and incorporated into the pharmaceutical industry for generation of potent chemotherapeutic drugs (Russo et al., 2010).

Dicerocaryum senecioides subsp. *transvaalense* (Klotzsch) J. Abels [family: Pedaliaceae], is a creeping perennial widely used both as a traditional medicinal plant and a nutritional source in many parts of southern Africa. The plant has been identified in our laboratory as a potential repository for anti-oxidant, anti-proliferative and anti-inflammatory agents. *Dicerocaryum senecioides* (vernacular: *malala 'a kwaetše or lempati*) grows widely in sandy soils of the veld in southern Africa. The sprawling stems grow vigorously in summer and less for the rest of the year. The plant covers an area of up to 10 m^2, and the stems bear distinctive fruit with two spines on the upper side. The small, hairy leaves of *D. senecioides* like those of related family members, *D. zanguebarium* (Lour.) Merrill and *D. eriocarpum* (Decne.) are used not only as food, but also in folk medicine for treating measles, as a hair shampoo, treatment of wounds, and to facilitate births in domestic animals and humans (Barone et al., 1995; Benhura & Marume 1992; Luseba et al., 2007). Nevertheless, the therapeutic mechanisms of action of *D. senecioides* have not been established. Recent findings in our laboratory have demonstrated that a dichloromethane extract of *D. senecioides* leaves exhibit strong anti-inflammatory (Madiga et al., 2009) and noticeable anti-proliferative properties against various cancer cell lines tested (unpublished data). These activities are assumed to be due to the plant's inherent anti-oxidative capacity, thought to be related to an abundance of phenolic compounds and flavonoids.

Exposure to potentially damaging reactive oxidants occurs unrelentingly throughout life because of the continuous endogenous generation of these agents by physiological processes, particularly mitochondrial respiration (Circu & Aw, 2010). Exogenous sources of reactive oxidants include cigarette smoke and other atmospheric pollutants, as well as certain pharmacological agents and chemicals. In addition to these, the phagocytic cells of the innate host immune system are also major producers of toxic oxidants. Neutrophils and macrophages are known to recruit and play vital roles in acute and chronic inflammation, respectively (Kasama et al., 1993). During inflammation, a marked recruitment and activation of inflammatory cells including neutrophils is noted. Activation of these phagocytes leads to the generation and release of reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide and hypochlorous acid (Weiss & Lobuglio, 1982). Depending on their concentration, ROS can be beneficial or damaging to cells and tissues. At physiological levels, ROS function as "redox messengers" in intracellular signaling and regulation, whereas excess ROS induce oxidative modification of cellular macromolecules, inhibit protein function, and promote cell death (Circu & Aw, 2010). Moreover, the generated ROS can influence the carcinogenic process by oxidatively damaging DNA and promoting malignant transformation in bystander cells in tissue culture (Jackson et al., 1989).

Since the identification of oxidant and antioxidant compounds is imperative for predicting and reducing health risks (Tunòn et al., 1995), while the identification and development of

useful and safe cancer treatment agents from herbal medicines is a well-recognised strategy in drug development (de Mesquita et al., 2009, 2011), this study was designed to investigate potential antioxidant and anti-proliferative activities of the leaf extracts of *D. senecioides* in order to corroborate its indigenous medicinal usage and the supposed indigenous exploit as a nutritional supplement.

2. Materials and methods

2.1 Materials used

All reagents were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated. All other reagents were of high quality and were obtained from reputable suppliers.

2.2 Methods

2.2.1 Extraction and fractionation

Fresh leaves of *D. senecioides* were collected, during the rainy season (January to April), from the grounds of the University of Limpopo, South Africa and dried for three days in an oven at 40°C. The dried leaves were then pulverised into a fine powder using pestle and mortar. The powder (46.15 g) was then extracted with absolute methanol (0.10 g/ml) by shaking for 72 h at room temperature. After the debris had settled, the green supernatant was filtered with a Whatman no.1 filter paper and concentrated to dryness under reduced pressure at 40°C using a Büchi Rotavapor R-200/205 (Büchi, Switzerland). The crude residue (14.79 g) was resuspended in ethanol: water (30:10, v/v). The ethanol: water extract was further fractionated by solvent-solvent extraction using *n*-hexane (D1 fraction), dichloromethane (D2 fraction), *n*-butanol (D3 fraction) and water (D4 fraction). The fractions were concentrated on a Büchi Rotavapor R-200/205 and the resultant residues (D1, 0.9 g; D2, 0.96 g; D3, 1.89 g; and D4, 5.4 g) were dissolved to an appropriate concentration in either acetone (for TLC) or dimethylsulphoxide (DMSO) (for anti-proliferative experiments) and stored in dark bottles at -20°C until required. A schematic representation of the fractionation procedure is illustrated in Fig. 1.

2.2.2 Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC). Ten microliters of each stock solution (10 mg/ml in acetone) from crude, D1, D2, D3 and D4 fractions were loaded individually onto the baseline of the Merck silica gel 60-F_{254} TLC plate (Macherey-Nagel, Düren, Germany) and the components were then separated with either chloroform: ethyl acetate: formic acid, CEF (10:8:2, v/v/v) (intermediate polarity/acidic) or butanol: acetic acid: water, BAW (4:1:5, v/v/v) (acidic/polar) or ethyl acetate: methanol: water, EMW (10:1.35:1, v/v/v) (polar/neutral) depending upon the nature of the fraction components. Chromatograms were visualised and circled under UV light at 254 nm and 365 nm for quenching and fluorescing compounds, respectively. The constituent phytochemicals were detected with vanillin-sulphuric acid reagent spray (0.1 g vanillin: 28 ml methanol: 1 ml sulphuric acid), followed by heating at 110°C in an oven for 3 min for optimal colour development.

2.2.3 Antioxidant activity analyses

The role of free radical reactions in disease pathology (e.g., in atherosclerosis, inflammation, ageing, ischemic heart diseases, neurodegenerative diseases, etc.) is well established, suggesting that these reactions are necessary for normal metabolism but can be detrimental to health as well. In this study, the antioxidant and free radical scavenging activities of the leaf extracts of *D. senecioides* were evaluated both qualitatively and quantitatively using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) chemical antioxidant assay.

2.2.3.1 TLC-DPPH antioxidant screening

The TLC-DPPH antioxidant assay is a qualitative method generally used for the screening of the anti-oxidative potential of plant extracts. It involves the chromatographic separation of the fractions, after which the chromatogram is sprayed with a purple/violet coloured radical solution, 0.2% DPPH (in absolute methanol). When DPPH interacts with antioxidant compounds, it accepts either electrons or hydrogen atoms, and this neutralises its free radical character. In the process, the purple/violet diphenyl-picrylhydrazyl colour is changed to a yellow diphenyl-picrylhydrazine colour in the presence of an antioxidant compound. The R_f values of the antioxidant compounds can thus be determined on the chromatogram. In this experiment, the TLC was run to separate the constituent compounds in the fractions, as described in section 2.2.2, and the plate was later stained with 0.2% DPPH solution to identify compounds that possess antioxidant activity.

2.2.3.2 DPPH radical scavenging effect

This is a quantitative assay method. Used as a reagent, DPPH offers a convenient and accurate method for titrating the oxidisable groups of natural or synthetic antioxidants. The 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activities of the crude, D1, D2, D3 and D4 fractions were determined according to the method described by Katsube et al. (2004). The assay involves the measurement of the disappearance of the coloured free radical, DPPH, by spectrophotometric determination. A 1000 µg/ml stock solution of the plant extract was serially diluted and pipetted (10 µl) into a 96-well plate. An equivalent amount of 100x diluted ascorbic acid (vitamin C) was used as a positive control. One hundred and eighty five microliters of a 0.2% DPPH solution (in absolute methanol and further dissolved in a 50% ethanol solution) was added to each well and the plate gently shaken for 5 min at room temperature. The change in absorbance at 550 nm was measured using a microtiter plate reader (DTX 880 multimode detector, Beckman Coulter, Fullerton, CA, USA). The percentage of scavenging activity was measured as:

$$\% \text{ inhibition} = \frac{[\text{absorbance of blank sample} - \text{test sample}]}{[\text{absorbance of blank sample}]} \times 100$$

Where, the blank sample contained only DMSO and DPPH.

2.2.4 Reducing power

Reducing powers of the crude, D1, D2, D3 and D4 fractions were measured using a spectrophotometric method. Briefly, various concentrations of the fractions (0-250 µg/ml) were prepared in 100 µl of dH_2O; vitamin C (100 µl of 100x dilute) was used as a positive control. Two hundred and fifty microliters of 0.2 M phosphate buffer (pH 6.6) and 1% (w/v)

potassium ferricyanide [$K_3Fe(CN)_6$] were added to each sample. The test samples were incubated at 50°C for 20 min. Two hundred and fifty microliters of 10% (w/v) TCA were later added to each tube and centrifuged at 900 x *g* for 10 min. Subsequently, 250 µl of the upper layer from each test sample was aspirated and transferred into clean Eppendorf tubes, and then diluted with an equal volume of dH_2O. An additional 50 µl of 0.1% (w/v) ferric chloride ($FeCl_3$) was added to each tube, mixed and 200 µl of this mixture transferred into a microtiter plate and the absorbance measured at 700 nm using a microtiter plate reader (DTX 880 multimode detector, Beckman Coulter, Fullerton, CA, USA).

2.2.5 Neutrophil isolation

In order to assess the selective inhibitory ability of the D2 fraction for the transformed cell lines, its cytotoxic effect was evaluated against the normal human neutrophil cells. Neutrophils were isolated from heparinised venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. The neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 x *g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Following centrifugation (280 x *g* at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), as determined by light microscopy and fluorescence microscopy (exclusion of ethidium bromide) respectively, were resuspended to (1×10^7 cells/ml) in PBS, pH 7.4, and held on ice until used.

2.2.6 Neutrophil viability

Isolated neutrophils ($1x10^7$ cells/ml) were exposed to the D2 fraction (0, 100, 200, 400, 600 and 800 µg/ml) for 0, 15 and 30 min at 37°C before addition of propidium iodide (PI). The percentage cells, whose cell membrane integrity was compromised (PI positive), were determined by using an Epics® Altra™ Flow Cytometer (Beckman Coulter Inc., Fullerton, CA, USA) fitted with a water-cooled Enterprise laser. PI intercalates into the DNA, and cannot enter the cell if the cell membrane is intact; therefore all PI positive cells have a disrupted cell membrane, indicating that these cells are not viable.

2.2.7 Cell culture

A murine macrophage cell line, Raw 264.7 (ATCC, Rockville, USA), was routinely cultured in RPMI-1640 growth medium (Gibco, Auckland, New Zealand) supplemented with 10% (v/v) fetal bovine serum, FBS (Hyclone, Cramlington, UK) and 1% (v/v) penicillin, streptomycin, neomycin (PSN) antibiotic cocktail (Gibco, Auckland, New Zealand) at 37°C in a humidified 5% CO_2/95% atmosphere.

2.2.8 MTT cytotoxicity assay

Cell viability of D2-treated Raw 264.7 cells was determined by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Experimental cells were seeded at 2×10^5 cells/ml in a 96-well cell culture plate (Nunc™, Roskilde, Denmark) and

incubated at 37°C overnight to allow the cells to attach. The cells were then exposed to various concentrations (0, 50, 100, 200, and 250 µg/ml) of the D2 fraction dissolved in DMSO. A mock sample (cells, RPMI-1640 and 0.05% DMSO) served as a negative control. The plates were incubated at 37°C for 0, 24, 48 and 72 h after which 40 µl of 5 mg/ml MTT was added to each well. Following incubation at 37°C for 3 h, the medium was aspirated and the remaining cells were washed once with pre-warmed PBS, pH 7.4. The reduced MTT was then dissolved in 50 µl DMSO and the absorbance was measured at 595 nm using a Model 550 microtiter-plate multimode detector (Bio-Rad Laboratories, California, USA). The percentage of viable cells was calculated as follows:

$$\% \text{ viability } = \frac{[A_{595}\text{of control } - A_{595}\text{of sample}]}{A_{595}\text{of control}} \times 100$$

2.2.9 Cell proliferation by real time cell analysis

The effect of the D2 fraction on cell proliferation was also assessed by real time cell analysis in which RAW 264.7 mouse macrophages were cultured for 24 h at 37°C in a 16-well E-plate 16 docked in a real time cell analysis dual plate (RTCA-DP) analyser (ACEA Biosciences, Inc., California, USA). After 24 h of growing, the cells were then treated with the D2 fraction as described in section 2.2.8 and cell densities of the treated cells were quantitatively monitored for 48 h at 37°C and represented as cell-index values.

2.2.10 Morphological analysis of apoptosis

The effects of the D2 fraction were evaluated for pro-apoptotic potential by microscopic analysis of the chromosomal DNA of the D2-treated Raw 264.7 cells. Apoptotic nuclei are identified by condensed chromatin gathering at the periphery of the nuclear membrane or totally fragmented apoptotic bodies. Briefly, cells were exposed to various concentrations of the D2 fraction (0, 50, 100, 200, and 250 µg/ml) for 24 h and washed with PBS, pH 7.4. The cells were then stained with 4', 6-diamidino-2-phenylindole (DAPI), which forms fluorescent complexes with double-stranded DNA by binding in the minor groove of the nucleic acid backbone. Cells were viewed and recorded under a Nikon Eclipse Ti inverted fluorescence microscope fitted with a camera (Nikon, Japan).

2.2.11 Statistical analysis

The results of each series of experiments are expressed as the mean values ± standard error of the mean (SEM). Levels of the statistical significance are calculated using the paired student *t*-test when comparing two groups, or by analysis of variance (ANOVA). The p-values of ≤ 0.05 were considered significant.

3. Results

3.1 Extraction and fractionation

The schematic representation of the extraction and the sequential (solvent-solvent) fractionation of the crude methanolic leaf extract of *D. senecioides*, using different solvents, are represented in Fig. 1.

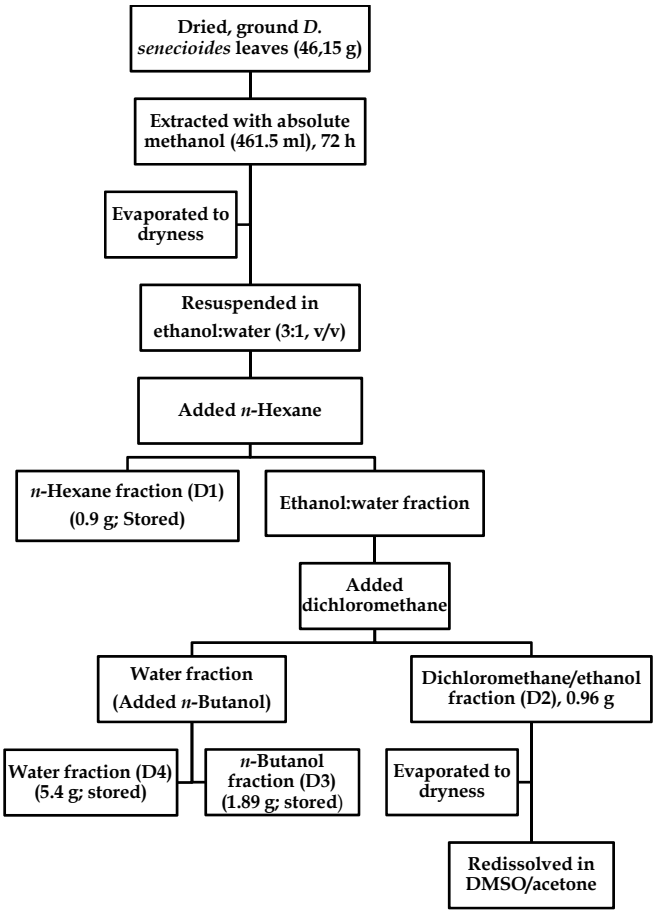


Fig. 1. The schematic representation of the fractionation of the crude methanolic leaf extract of *D. senecioides*.

3.2 TLC-DPPH free radical scavenging activity

TLC was used for the qualitative detection of constituent compounds in *D. senecioides.* As shown in Fig. 2A, the D2 fraction showed the most constituent components with strong intensities using the CEF (10:8:2, v/v/v), EMW (10:1.35:1, v/v/v) and BAW (4:1:5, v/v/v) solvent systems followed by the crude, and then the D3 fraction. Both the D1 and D4 fractions did not exhibit any noticeable antioxidant activity on the TLC plate. Since the antioxidant activity of plant extracts cannot be evaluated by only one method because of the complex nature of phytochemicals, it was thus imperative to use several assays to evaluate the antioxidant activity of of *D. senecioides* leaf extracts. The TLC-DPPH method of qualitative antioxidant detection revealed that crude, D2 and D3 fractions all possessed antioxidant activities which were manifested by a bright yellow colour. The three fractions exhibited a similar R_f value (R_f = 0.78) when resolved on the BAW solvent system.

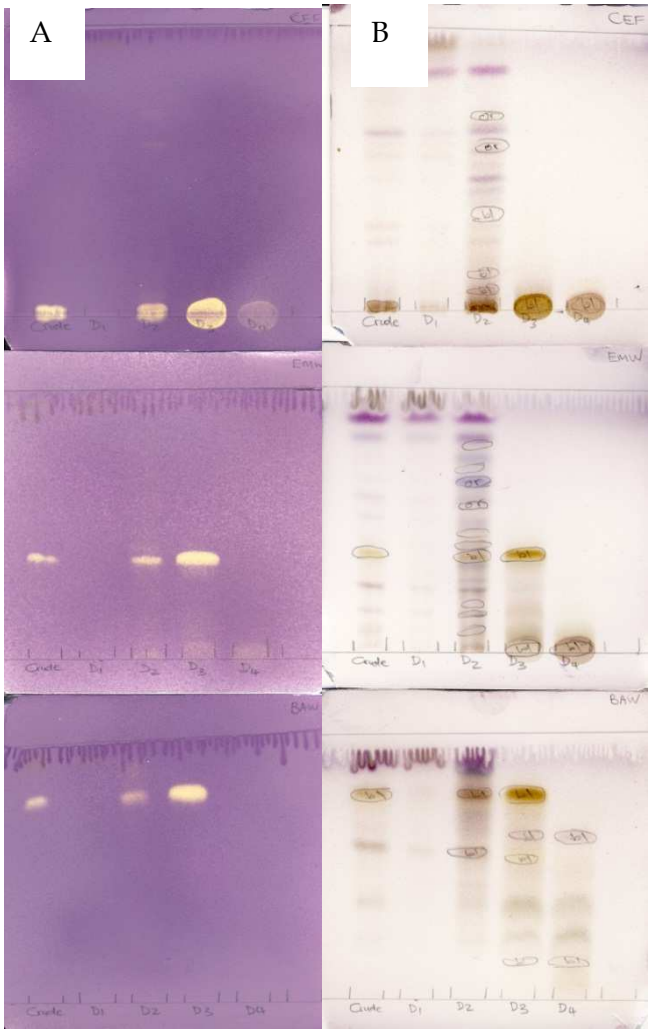


Fig. 2. Chromatograms of the various fractions of *D. senecioides* sprayed with vanillin/ sulphuric acid reagent (A) to show compounds extracted with methanol (crude), *n*-hexane (D1), dichloromethane (D2), *n*-butanol (D3) and water (D4); and 0.2% DPPH solution (B) to indicate compound(s) with antioxidant activity. Note the high antioxidant activity displayed by the D3 fraction, and the moderate amounts in the crude and D2 fractions. Both D1 and D4 fractions exhibited no detectable antioxidant activity.

The D3 fraction, in particular, had the strongest scavenging activity (Fig. 2B). These observations demonstrate the presence of antioxidant compounds in *D. senecioides*. Because this is a qualitative method, a quantitative assay was also used to evaluate the abilities of the fractions to scavenge DPPH.

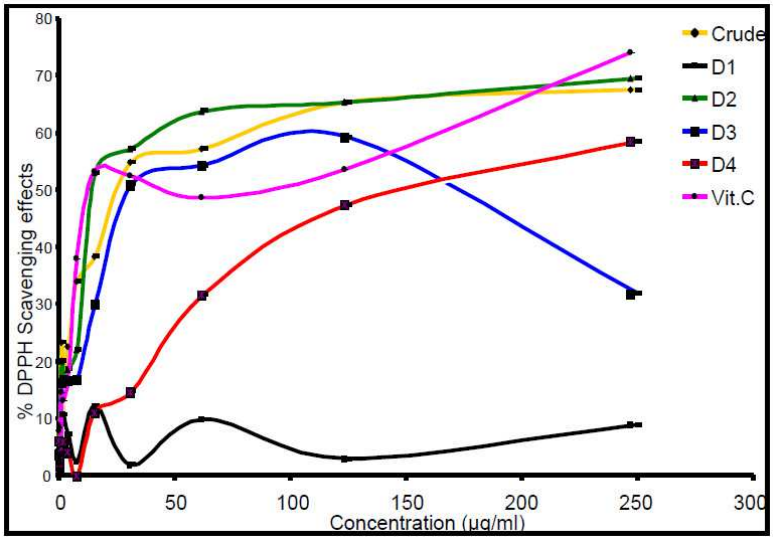


Fig. 3. DPPH radical scavenging capacities of methanol (crude), *n*-hexane (D1), dichloromethane (D2), *n*-butanol (D3) and water (D4) fractions.

3.2 DPPH free radical scavenging activity

The anti-oxidative potential of the D2 fraction was also evaluated quantitatively using the DPPH free radical scavenging activity assay. As shown in Fig. 3, the D2 fraction possessed a high DPPH-scavenging activity from 62.5 µg/ml (63.8%) to 250 µg/ml (69.5%) as compared to the other fractions; the order of potency in descending order was D2> crude> D3> D4> D1 (Fig. 3). Vitamin C (diluted 100x) was used as a positive control. DMSO, as a negative control, showed no scavenging activity. The reducing powers of the fractions were also evaluated.

3.3 Reducing ability

The reducing potentials of the different fractions were determined by their ability to reduce ferric ions. The presence of antioxidants with reducing potential causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous (Fe^{2+}) form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Öztürk et al., 2006). Fig. 4 shows the reducing power of *D. senecioides* fractions and vitamin C (positive control) using the potassium ferricyanide reduction method. The reducing power of the fractions increased with increasing concentrations. The D2 fraction showed a higher reducing power than the other fractions in a dose-dependent fashion. However, its reducing power was less than that of vitamin C which was diluted 100x.

3.4 Neutrophil viability

Since the D2 fraction displayed the most potent anti-oxidative activity, it warranted further assessment on the possible anti-proliferative activity against cancer cells. Thus, its selective cytotoxicity for the transformed cell lines was investigated against freshly isolated human

blood neutrophils, used as normal control cells. The neutrophils, treated with the D2 fraction for 15 min, showed a significant decrease in viability at a concentration of 400 µg/ml with 91% viable cells; while after 30 min, a significant decrease was observed at 600 µg/ml with 89% viable cells (Fig. 5). However, the experimental concentrations used (0-250 µg/ml) were found not to be cytotoxic to the normal, control neutrophil cells.

3.5 Anti-proliferative effect of D2 fraction on RAW 264.7 cells

The effect of the D2 fraction on proliferation of RAW 264.7 cells was analysed by determining viability and growth of treated cells using the MTT assay and real time cell analysis, respectively. The D2 fraction decreased cell viability (Fig. 6) and growth (Fig. 7) in a time- and dose-dependent manner.

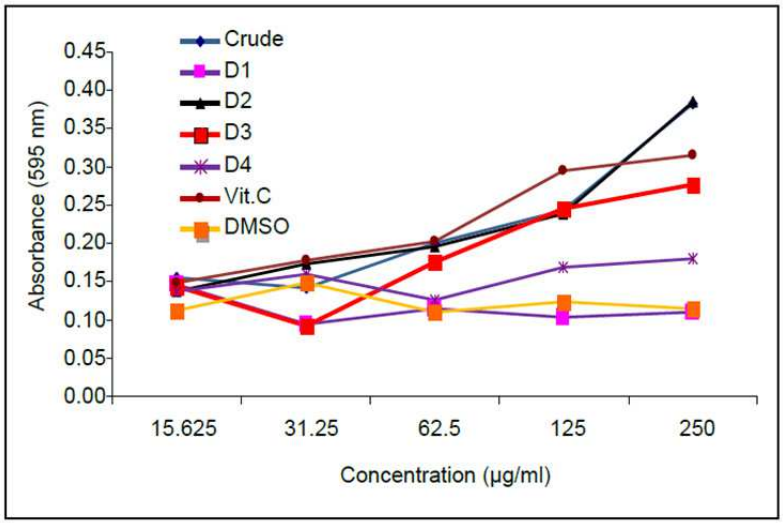


Fig. 4. Reducing powers of methanol (crude), *n*-hexane (D1), dichloromethane (D2), *n*-butanol (D3) and water (D4) fractions, vitamin C (positive control, 100x diluted) and DMSO (negative control, less than 0.1%).

3.6 Apoptotic morphology

Treated cells were shown to die through apoptosis when stained with DAPI nucleic acid stain (Fig. 8). The DAPI stain is sensitive to DNA conformation and the state of chromatin in cells, and is thus used to grade nuclear damage. Cells dying through apoptosis displayed nuclei that stained bright blue as their chromatin was condensed, whilst those that had reached advanced stages of apoptosis showed totally fragmented morphology of nuclear (apoptotic) bodies that also stained bright blue (Fig. 8).

4. Discussion

The objective of this study was to investigate the anti-oxidative and anti-proliferative potentials of leaf extracts of *D. senecioides*. TLC serves as a qualitative, screening method to

characterise the phytochemical constituents of plants. It also enables comparison of the chemical composition of different fractions using different solvents; high quality resolution is based on the polar/nonpolar nature of the constituent compounds. TLC separation of the fractions showed that the D2 fraction contains more compounds when sprayed with vanillin than the other fractions (Fig. 2A).

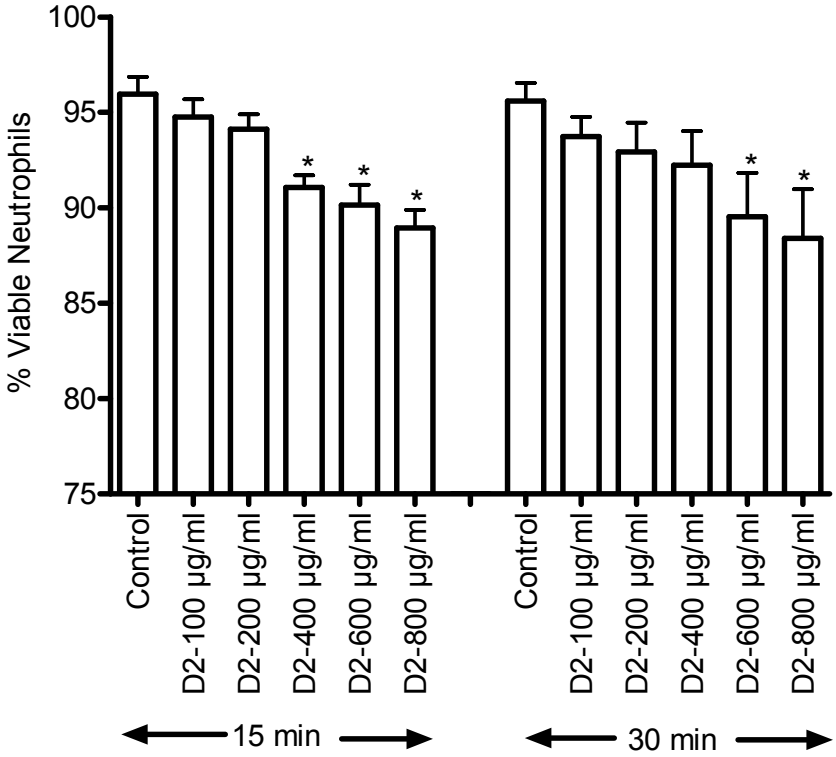


Fig. 5. Viability of normal human neutrophils after exposure to various concentrations of the D2 fraction for 0, 15 and 30 min at 37ºC before addition of propidium iodide (PI). The results represent five different experiments expressed as mean values ± SEM. *Statistically significant (P<0.05).

Subsequent investigations were focused on the anti-oxidative potential of the D2 fraction. Oxygen-derived free radicals and other reactive oxygen species (ROS) generated endogenously and exogenously are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and the ageing process (Halliwell & Gutteridge, 1999). Many natural products are available as chemoprotective agents against common cancers worldwide, and many of these are powerful antioxidants. These natural products, many of which are phenolic compounds, are found in vegetables, fruits, plant extracts and herbs. Although the mechanism of their protective effects is unclear, the fact that the consumption of fruits and vegetables lowers the incidence of carcinogenesis is broadly supported (Reddy et al., 2003). Certainly, anti-oxidative properties are generally considered to confer beneficial, chemopreventive properties on a molecule.

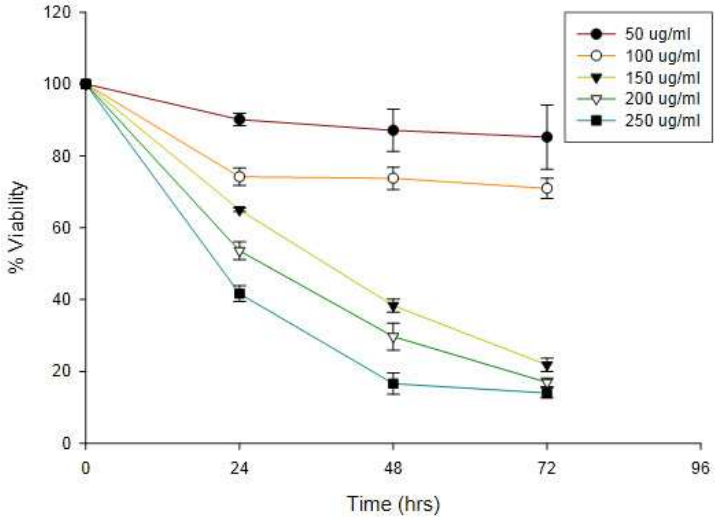


Fig. 6. The effect of D2 fraction on the viability of RAW 264.7 cells. Cells were treated with increasing concentrations of the D2 fraction for 0, 24, 48 and 72 h, followed by viability determination by the MTT assay. Viability was calculated as percentage of untreated control cells. The results represent the mean of two independent experiments, each done in duplicate ± SEM.

The antioxidant activities of the various fractions were measured using qualitative TLC-DPPH and a quantitative DPPH spectrophotometric assay based on the scavenging of the stable DPPH free radical, while the Fe^{3+}-Fe^{2+} reductive method was used to determine the reducing potentials of the fractions. Different antioxidant assays are used to facilitate the screening and identification of the anti-oxidative activity of plant fractions in comparison with that of known, stable antioxidants. Because antioxidants can act by different mechanisms, and more than one mechanism can be involved, it is possible for an antioxidant to protect in one system, but fail in another. Evidently, the understanding of mechanisms and dynamics of the antioxidant action is essential for designing appropriate experimental methods and proper interpretation of the results. Therefore, antioxidant activity must be evaluated using different test methods on the basis of the mechanisms and dynamics of antioxidant action (Arouma, 2003; Niki, 2010).

Consequently, the free radical scavenging activities of the plant fractions were evaluated according to their abilities to scavenge synthetic DPPH. This assay provides useful information on the reactivity of the compounds with stable free radicals. Vitamin C was used for comparison because it is known to be the most abundant and effective water-soluble antioxidant in the body. The qualitative TLC-DPPH antioxidant method was used to screen fractions in order to indicate which of these had potential antioxidant activity which merited further investigation. After the plates were sprayed with the DPPH solution, unique bands with strong and characteristic intense yellow colour appeared (Fig. 2B). The D3 fraction showed a strong yellow colour followed by D2> crude> D1> D4 fractions. The intensity of the yellow colour depends on the quantity and nature of the compound present at that area.

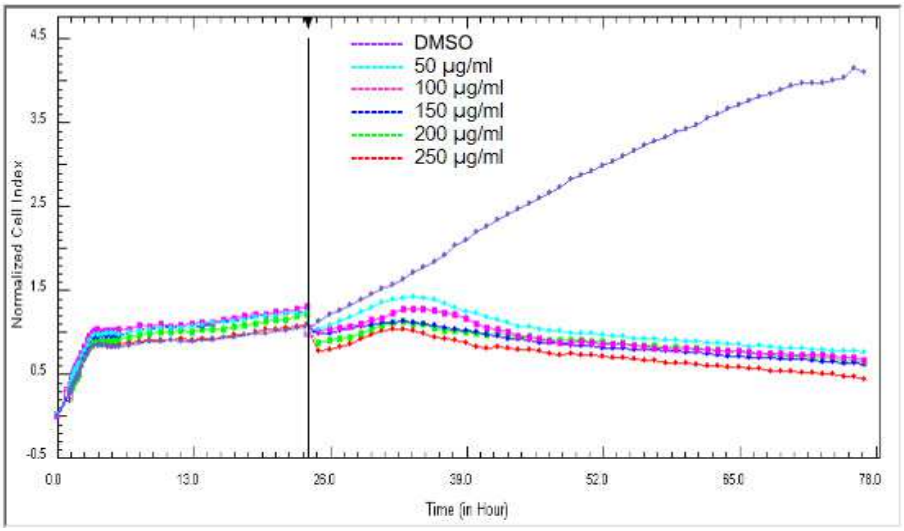


Fig. 7. Real time cell analysis plot of RAW 246.7 cells treated with the D2 fraction. Cells were cultured for 24 h and then followed by treatment with various concentrations of the D2 fraction for a further 48 h. Cell proliferation was measured by continuously monitoring cell indices using an RTCA-DP analyser. Cell indices were normalised at 24 h and then treated with the extract (see arrow head). The final DMSO concentration was 0.05%.

The quantitative DPPH spectrophotometric assay method was also used to quantify the antioxidant activities of the crude, D1, D2, D3 and D4 fractions. This is a rapid and low cost method commonly used in antioxidant studies. An antioxidant molecule present in plant extracts can quench the DPPH free radicals by either providing a hydrogen atom or by donating an electron (Bondet et al., 1997). The results of the quantitative DPPH assay suggest that the D2 fraction possesses an impressive antioxidant scavenging activity in comparison to D1, D3, and D4 (Fig. 3). Comparison of these results showed a contradictory relationship between the activities measured by the qualitative TLC-DPPH screening method and the quantitative DPPH spectrophotometric assay. The D3 fraction showed stronger scavenging activity in the qualitative TLC-DPPH assay (Fig. 2B) than the other fractions, while in the quantitative DPPH assay (Fig. 3), the D2 fraction displayed the strongest anti-oxidative activity (found to be comparable to the crude extract), possibly because of the synergistic behaviour of compounds present in the D2 fraction.

The aforementioned methods focused on the radical scavenging activities of antioxidants extracted from the fractions. However, the antioxidant activities of natural antioxidants may also result from their reducing powers, because the constituent compounds may act by donating electrons to free radicals and convert them to more stable products. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Rajeshwar et al., 2005). The D2 fraction showed significant reducing power when assayed using the potassium ferricyanide test (Fig. 4). This fraction also demonstrated the ability to convert $FeCl_3$ from the ferric to the ferrous state, a feature indicative of hydrogen-donating potential (Rajeshwar et al., 2005). The D2 fraction was therefore found to have various radical scavenging activities that could be due to the presence of a number of

phenolic compounds. Clearly, further work needs to be undertaken to confirm the antioxidant property of this fraction by using other antioxidant assessment methods, as well as to characterise and identify the active agent or agents.

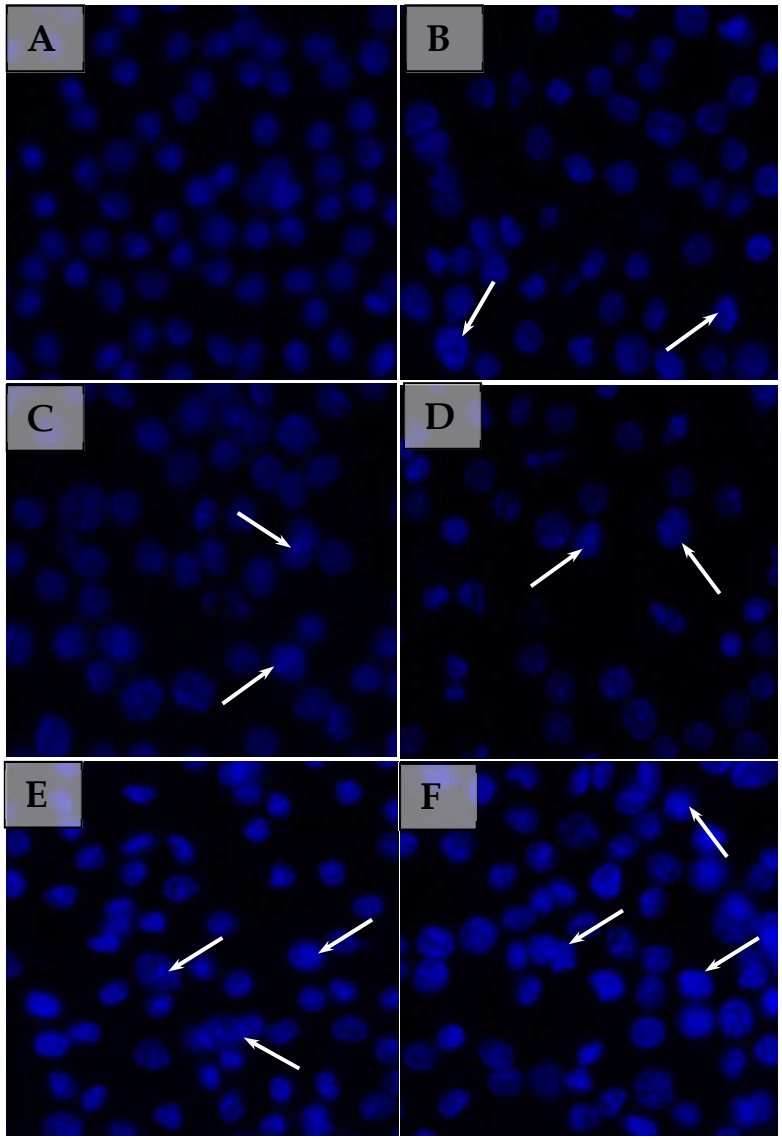


Fig. 8. DAPI nucleic acid staining demonstrating apoptotic morphology in RAW 264.7 cells after exposure to various concentrations of D2 fraction for 24 h. Cells were photographed under an inverted fluorescence microscope at 40x magnification. A = DMSO control, B = 50 µg/ml, C = 100 µg/ml, D = 150 µg/ml, E = 200 µg/ml and F = 250 µg/ml. Arrows indicate nuclear shrinkage and chromatin condensation.

The D2 fraction was further evaluated for anti-proliferative activity by analysis of its effect on the growth and viability of Raw 264.7 cells, a murine-derived macrophage cell line, using real time cell analysis and MTT assay, respectively. The MTT assay demonstrated that the D2 fraction induced a time- and concentration-dependent decrease in cell viability (Fig. 6). Fig. 7 showed that the D2 fraction resulted in a decrease in the cell index, a unit-less, quantitative measure of the number of cells present in a given E-plate 16 well. The measurement of cell proliferation using an RTCA-DP analyser allowed constant monitoring of cell death in real-time without interfering with cell behaviour. Further, evaluation of the effect of the D2 fraction in normal human neutrophil cells demonstrated a degree of specific and selective cytotoxicity to cancer cells. This was so because the viability of the normal neutrophil cells was unaffected by the experimental concentration range (0-250 µg/ml) used. However, these cells were found to be moderately susceptible to the toxic D2 concentrations (600 and 800 µg/ml) used (Fig. 5).

Furthermore, Raw 264.7 cells were shown to die through apoptosis as indicated by the staining of the treated cells with DAPI nucleic acid stain. Cells undergoing apoptosis are characterised by cytoplasmic shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Wyllie et al., 1980). Indeed, these hallmark features of apoptosis were observed in Raw 264.7 cells treated with various concentrations of the D2 fraction for 24 h (Fig. 8). In contrast, the chromatin of untreated cells remained intact and unaffected after 24 h, as indicated by the evenly spread DAPI nucleic acid stain and granular morphological appearance of the stained nuclei (Fig. 8). The pro-apoptotic activity of the D2 fraction and the prevention of excess free radical production by the D2 fraction illustrate a commendable chemoprotective and chemotherapeutic potential, as this ability allows the fraction to induce cancer cell death without eliciting the inflammatory response often associated with necrotic cell death.

Since bioactive compounds within plant extracts are often blended with other ineffectual constituents, and the activity of the compound of interest is frequently dampened or masked by these unwanted compounds, plant extracts require further purification in order to isolate the active ingredients in their pure form, and/or to trim down the redundant, interfering components. In this study, the D2 fraction was found to possess both anti-oxidative and selective anti-proliferative properties. It is, therefore, essential to fractionate and purify the D2 fraction and to evaluate the anti-oxidative and the anti-proliferative potentials of the resultant bioactive principles against positively tested anticancer compounds (such as doxorubicin) that are currently available in the market.

5. Conclusion

The D2 fraction of *D. senecioides* displayed properties of an ideal anti-oxidative and anti-proliferative agent. The fraction showed impressive antioxidant activity both as a free-radical scavenger and a reducing agent. Moreover, the induction of apoptosis in the macrophage lineage used suggests that the fraction has the potential to combat chronic inflammation at three stages: i.e., inhibiting initial free radical production, mopping up excess free radicals and eradicating macrophages responsible for the over-production of reactive oxygen species. Furthermore, the pro-apoptotic activity of the D2 fraction has the ability to induce cancer cell death without eliciting the inflammatory response observed in necrotic cell death. Future investigations should, therefore, focus not only on unravelling the

actual anti-oxidative and anti-proliferative mechanisms of action, but also on molecular and chemical characterisation of the active components of the D2 fraction. Irrespective of the underlying mechanism(s) of action and precise identification of the active chemical entities present in the D2 fraction, the results of the current study have established a promising anti-oxidative and anticancer potential of the D2 fraction of *D. senecioides*. Considering chemoprevention of cancer, as well as other disorders, particularly chronic inflammatory diseases, this represents a particularly attractive and promising combination of biological activities. Certainly, a full understanding of the redox control of apoptotic initiation and execution could also underpin the development of therapeutic intervention strategies targeted at other oxidative stress-associated disorders.

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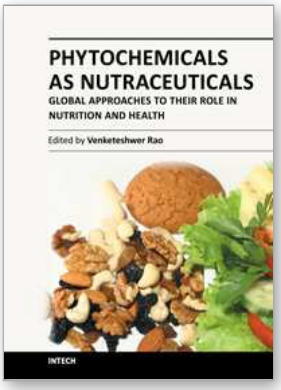
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Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health

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Phytochemicals are biologically active compounds present in plants used for food and medicine. A great deal of interest has been generated recently in the isolation, characterization and biological activity of these phytochemicals. This book is in response to the need for more current and global scope of phytochemicals. It contains chapters written by internationally recognized authors. The topics covered in the book range from their occurrence, chemical and physical characteristics, analytical procedures, biological activity, safety and industrial applications. The book has been planned to meet the needs of the researchers, health professionals, government regulatory agencies and industries. This book will serve as a standard reference book in this important and fast growing area of phytochemicals, human nutrition and health.

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