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Free radical scavenging activity from leaves of *Acacia nilotica* (L.) Wild. *ex* Delile, an Indian medicinal tree

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ABSTRACT

The present study compares the two extraction methods and evaluates the free radical scavenging activity of *Acacia nilotica*. Results indicated that the sequential extraction method was effective in concentrating the active principles in the ethanol extract as compared to the maceration method in DPPH assay. Based on the results, free radical scavenging property of the extracts obtained from sequential extraction method was analyzed in different assays to find out the possible antioxidant mechanism. Our results indicate that ethanol extract rich in phenolic and flavonoid contents had potent antioxidant activity and were significant in comparison with all the positive controls used in this study. The possible antioxidant mechanism of the ethanol extract can be due to its hydrogen or electron donating and direct free radical scavenging properties. Hence, the ethanol extract represents a source of potential antioxidants that could be used in pharmaceutical and food preparations.

1. Introduction

Free radicals have been shown to be harmful as they react with important cellular components such as proteins, DNA and cell membrane (Mantena et al., 2008). The body on the other hand, requires free radicals for immune system responses. However, an overload of these molecules has been linked to certain chronic diseases of heart, liver and some form of cancers (Prakash et al., 2007). All organisms contain anti-free radical defence system, which includes antioxidant enzymes like catalase, peroxidase and superoxide dismutase and antioxidants like ascorbic acid and tocopherol.

At present, there is special interest on natural antioxidants coming from the plant resources. There are more evidences suggesting that phytochemicals having antioxidant properties are associated with a lower risk of mortality from many of the diseases (Rice-Evans, 2004; Dixon et al., 2005). It has been reported that the antioxidant activity of plants might be due to their phenolic compounds, flavonoids, alpha-tocopherol and carotenoids (Geetha et al., 2005). Natural products have been shown to be a tremendous and consistent resource for the development of new drugs (Newman and Cragg, 2007).

Acacia nilotica is a multipurpose tree of Fabaceae family that is used extensively for the treatment of various diseases, e.g. cold, bronchitis, diarrhoea, dysentery, biliousness, bleeding piles and leucoderma (Ambasta, 1994). It is widely distributed in different regions of the world. It is used by traditional healers of different regions of Chhattisgarh in treatment of various cancer types of mouth, bone and skin. In West Africa, the bark and gum are used against cancers and/or tumors (of ear, eye, or testicles) and indurations of liver and spleen, the root for tuberculosis, the wood for smallpox and the leaves for ulcers.

In the present investigation, the leaves were chosen based on the previous reports. It was reported that the leaf extract of *A. nilotica* had significant chemo preventive and anti mutagenic activity than the other parts (Meena et al., 2006). The leaf powder of the plant was extracted with different solvents by sequential extraction method in the order of increasing polarity, and with ethanol alone by maceration method. The free radical scavenging property of each of the extracts obtained by both the extraction methods was compared in DPPH assay and thin layer chromatographic analysis. Based on the results, the free radical scavenging property of the extracts obtained from sequential extraction method was analyzed in different assays such as reducing power, lipid peroxidation and deoxyribose degradation (site-specific and non-site-specific) to find out the extract as hydrogen or proton donor or direct free radical scavenger or metal chelator. The total phenolic and flavonoid contents were also determined to find out the relationship between free radical scavenging assays and phenolic compounds.

Abbreviations: PE, petroleum ether; BZ, benzene; DCM, dichloromethane; CF, chloroform; EA, ethanol; AQ, water; TPC, total phenolic content; TFC, total flavonoid content; TBA, thio barbituric acid; RP, reducing power; DPPH, DPPH radical scavenging; NSSDRD, non-site-specific deoxyribose degradation; LPA, lipid peroxidation.

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2. Materials and methods

2.1. Plant material

The plants were collected from vicinity of VIT University, Vellore during the month of August 2007. It was identified and voucher specimen deposited at the High altitude Plant Physiology Research Center, Hemwati Nandan Bahuguna Garhwal University, India.

2.2. Preparation of extracts

2.2.1. Maceration extraction method

For maceration extraction, air-dried *A. nilotica* leaves were soaked with ethanol in an Erlenmayer flask, and was left to macerate in the dark for 5 days at room temperature. The filtered ethanolic extract was dried over vacuum desiccator and the solvent was removed in vacuum.

2.2.2. Sequential extraction method

Air-dried *A. nilotica* leaves were packed into a soxhlet apparatus and was extracted sequentially with petroleum ether (PE), benzene (BZ), dichloromethane (DCM), chloroform (CF), ethanol (EA) and water (AQ). The organic extracts were dried over vacuum desiccator and the solvent was removed in vacuum. The extracts were dissolved in dimethyl sulfoxide (DMSO), ethanol or water prior to analysis depending upon their solubility. The extracts were subjected to further analysis and all the assays were done in triplicates.

2.3. Thin layer chromatography (TLC)

Aliquots of ethanol extracts obtained from both extraction methods were loaded on the activated silica gel TLC plates (20 cm × 20 cm). The plates were developed using ethyl acetate:toluene:formic acid (3.5:3:0.5). The spots were located by exposing the plate to UV light and iodine fumes.

2.4. Determination of phytoconstituents

2.4.1. Determination of total phenolics

The total phenolic content (TPC) of different extracts of *A. nilotica* was determined by the method of Folin–Ciocalteu reaction (Kujala et al., 2000), using gallic acid as standard. To the extract, Folin–Ciocalteu reagent and Na_2CO_3 was added. After 20 min incubation at room temperature, the absorbance was measured at 730 nm.

2.4.2. Determination of total flavonoids

The total flavonoid content (TFC) of the different extracts of *A. nilotica* was determined by slightly modified method (Nieva Moreno et al., 2000). To the extract, potassium acetate and aluminium nitrate was added. After 40 min incubation at room temperature, the absorbance was measured at 415 nm using quercetin as standard.

2.5. Antioxidant testing assays

2.5.1. Determination of reducing power (RP)

The iron (III) reductive capacity was assessed as described by Oyaizu (1986). Briefly, 1 ml of extract in an appropriate solvent was mixed with phosphate buffer and $K_3Fe(CN)_6$ solution. After 30 min at 50 °C, 10% TCA was added and the mixture was centrifuged for 10 min at 2000 rpm. Finally, a 2.5 ml aliquot was mixed with 2.5 ml ultra-pure water and 0.5 ml of 0.1% $FeCl_3$, the absorbance was recorded at 700 nm, and the results were represented as ascorbate equivalents.

2.5.2. 1,1-Diphenyl-2-picrylhydrazyl free radical scavenging activity (DPPH)

The ability of the extract to scavenge DPPH radicals was assessed as described by Ohinishi et al. (1994). To the different concentrations of extract, 3 ml of freshly prepared ethanolic DPPH (0.1 mmol/l) solution was added. After 30 min of incubation in dark, the absorbance was recorded at 517 nm. Results were expressed as percentage inhibition of DPPH.

$$\%\text{Inhibition} = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)/\text{Abs}_{\text{control}}\right] \times 100$$

The percentage inhibition was plotted against the sample extract concentration in order to calculate the IC_{50} values, which is the concentration (μg/ml) of extract that causes 50% loss of DPPH activity. Results were compared with the positive controls, ascorbic acid, quercetin, tocopherol and catechin.

2.5.3. Lipid peroxidation by thiobarbituric acid (TBA) assay

Lipid peroxidation activity of extracts was carried out by modified method of Ohkawa et al. (1979). TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. Fish livers were used for the preparation of liver homogenate. The homogenate was centrifuged at 12000*g* for 15 min at 4 °C, and the supernatant was used for *in vitro* lipid peroxidation assay. The absorbance was measured at 535 nm. (+)-catechin was used as a positive control. The percentage inhibition of lipid peroxidation is calculated as in DPPH assay.

2.5.4. Hydroxyl radical scavenging effect

2.5.4.1. Non-site-specific deoxy ribose degradation assay (NSSDRD). Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Kunchandy and Rao (1990). The free radical damage imposed on the substrate, deoxyribose was measured as TBARS by the method of Ohkawa et al. (1979). The absorbance was measured at 532 nm against control containing deoxyribose and buffer. Catechin was used as a positive control. The percentage inhibition was determined as in DPPH radical scavenging assay.

2.5.4.2. Site-specific deoxy ribose degradation assay (SSDRD). The ability of the extracts to inhibit site-specific hydroxyl radical-mediated peroxidation was carried out as described in the non-site-specific hydroxyl radical-mediated peroxidation inhibition procedure, except that EDTA was replaced by buffer (Evans et al., 1997).

2.6. Statistical analysis

All experiments were repeated at least three times. Results were reported as mean ± standard error. The statistical significance between antioxidant activity values of the extracts was evaluated with one way ANOVA followed by Holm-Sidak test. *P* values less than 0.05 were considered to be statistically significant. Correlation analyses between different antioxidant assays and between the total phenolic content and total flavonoid content were carried out using the correlation programme in MINITAB.

3. Results and discussion

3.1. Extraction method and TLC analysis

For solvent extraction, we first investigated extracting the leaves of the plant using maceration method, with ethanol as a solvent, but little radical scavenging activity was observed with the DPPH assay (IC_{50} = 45 μg/ml). We then investigated the sequential extraction method using six different solvents in the order of increasing polarity viz. petroleum ether, benzene, dichloromethane, chloroform, ethanol and water (Flow chart 1). The same ethanol extract after sequential extraction showed high free radical scavenging activity (IC_{50} = 6.5 μg/ml); therefore we reported this method only. In this study, six solvents were employed to determine the most effective extraction solvent having active principles. The efficacies of the solvents were analyzed in different free radical scavenging assays. Non-polar solvents like petroleum ether, benzene and dichloromethane showed little activity in our assays. Thus, the sequential extraction of the plant with non-polar solvents aided the removal of most of the interfering substances thereby concentrating the active principles in the ethanol extract.

The ethanol extract obtained from both extraction methods were analyzed in TLC and both showed unique pattern of chromatogram. Photograph 1a shows some compounds (orange spots) found in the extract of maceration method are not found in the one obtained from sequential extraction method indicating that these compounds have interfered the free radical scavenging activity. Photograph 1b reveals some compounds are in more concentrated form in the extract obtained from sequential extraction method than the one obtained from maceration method indicating that these compounds could be responsible for the antioxidant activity. This could explain the efficacy of the sequential extraction procedure that is involved in the removal of most of the interfering substances. This method also simplifies further fractionation process that leads to the identification of active principles, which is under progress.