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Antioxidant potential of different extracts and fractions of *Catharanthus roseus* shoots

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Abstract

Antioxidant effectiveness of indigenous medicinal plant C. roseus shoots extracts and fractions with solvents of different polarity (n–hexane, ethylacetate, methanol, chloroform) was assessed for total phenolics content (TPC), total flavonoid contents (TFC), DPPH radical scavenging activity and % inhibition of peroxidation in linoleic acid system. The *C. roseus* extracts and fractions contained appreciable levels of total phenolic contents 3.2 to 8.5 GAE (g/100g per dry matter) and total flavonoid contents 1.8 to 5.4 CE (g/100 of per dry matter). The C. roseus shoots extracts and fractions also exhibited good DPPH radical IC_{50}, 28.2 to 119 µg/ml and Inhibition of Peroxidation in Linoleic Acid (38.4 to75.1%) respectively. Of the *C. roseus* shoots extracts and fractions tested, 100% methanolic extract exhibited the maximum antioxidant activity, the results of the present investigation demonstrated significant ($p<0.05$) variations in the antioxidant activity. The results of the present comprehensive analysis demonstrated that *C. roseus* extracts and fractions are a viable source of natural antioxidants and might be exploited for functional foods and nutraceutical applications.

Introduction

Antioxidants are radical scavengers which give protection to human body against free radicals by inhibiting the oxidizing chain reactions. When these substances are present at low concentration in body they markedly delay or prevent the oxidation of an oxidizable substrate [16]. These antioxidants always play important roles in delaying the development of chronic diseases such as cardiovascular diseases (CVD), cancer, atherosclerosis, inflammatory bowel syndrome and Alzheimer's diseases [4].

Catharanthus roseus or Vinca rosea belongs to the family Apocynaceae, (Genus, catharanthus) the other common names are periwinkle, madagascar periwinkle, sadabahar. The plant can grow easily and is commonly available in the sub-continent. *Catharanthus roseus* has a variety of medicinal properties, such as antibacterial [2], antifungal [9] and antiviral [6]. A variety of different alkaloids is present in C. roseus: more than 130 different compounds have been reported including about 100 monoterpenoid indole alkaloids [12]. As an important medicinal plant, it has a good antioxidant potential throughout its parts under drought stress. All parts of the plant are rich in alkaloids but root bark contains maximum concentrations of it particularly during flowering [8].

In spite of all the attention focused on C. roseus, determination of natural products other than alkaloids in this plant remains scarce. A few recent reviews [11&13] about the existence of

phenolics illustrate that little has been done in the characterization of this group of compounds in *C. roseus* and, moreover, most of the work reported has been done with cell cultures, where the metabolism of natural products is quite poor compared with the differentiated plant body. Study of noncolored phenolics in *C. roseus* has not been done. Little information is also available about the antioxidant potential of *C. roseus*. Previous studies concerned the evaluation of the influence of environmental factors on the antioxidant defense system of the plant, namely antioxidant enzymes and indole alkaloids [8 &10].

Zheng and Wang [18] reported the oxygen radical absorbance capacity (ORAC) of crude extracts of several culinary and medicinal herbs including *C. roseus*, which displayed one of the highest capacities among the medicinal species.

The objective of this study was to determine the antioxidant potential of different fractions of *C. roseus* shoots in solvents of different polarity by a series of protocols using some biologically relevant models.

Materials and methods

Plant material

The selected plant *Catharanthus roseus* shoots were collected from the Botanical Garden, University of Agriculture, Faisalabad, and further identified from the Department of botany University of Agricultural Faisalabad, Pakistan where a voucher specimen number has been deposited.

Preparation of Extracts and different fractions.

3Kg fresh shoots of *C. roseus* plant washed with distilled water so that the adhering dust particles must be removed. They were dried in the shaded place. The dried leaves were powdered and stored in the clean containers. In the weighed amount of powdered leaves the measured amount of 100% methanol (2×15 L) was added and kept it for 4-5 days at room temperature. The solvent was removed by using rotary evaporator. Extract (140 g) became viscous which were dried on water bath and then stored at – 4 ^{0}C. The process was repeated three times with intervals of four days. The methanolic extract was dissolved in distilled water and fractionation was done by using different polarity based solvents and got n-hexane (50g), 100% chloroform (42g), 100% ethyl acetate (40g) fractions successively. The 100% ethylacetate fraction (E) was subjected to column chromatography over a flash silica gel eluting with ethylacetate and gradient of chloroform upto 100%. Four fractions (E1 – E4) were collected. The same procedure was repeated with remaining residue but solvents used for elution were methanol and ethylacetate with varied polarity and three fractions (M1- M3) were collected as shown in Fig 1. All fractions and extracts obtained were investigated for antioxidant activity using different assays.

Antioxidant assay

Determination of total phenolics (TP)

Amount of TPC were assessed using Folin-Ciocalteu reagent [3]. Briefly, 50mg of crude extract was mixed with 0.5mL of Folin-Ciocalteu reagent and 7.5mL deionized water. The mixture was kept at room temperature for 10 min, and then 10mL of 20% sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40^{o}C for 20 min and then cooled in an ice bath; absorbance at 755nm was measured using a spectrophotometer (U-2800, Hitachi Instruments Inc., Tokyo, Japan). Amounts of TP were calculated using gallic acid calibration curve within range of 10-100 ppm (R^2 = 0.9986). The results were expressed as gallic acid equivalents (GAE) g/100g of dry plant matter. All samples were analyzed thrice and results averaged. The results are reported on dry weight basis.

Determination of total flavonoids (TF)

The TFC were measured by a spectrophotometric method following a previously reported method by Dewanto et al [5]. Briefly, extract of each method (1mL containing 0.1mg/mL) was diluted with 4mL water in a 10mL volumetric flask followed by 0.3 mL of 5% $NaNO_2$.

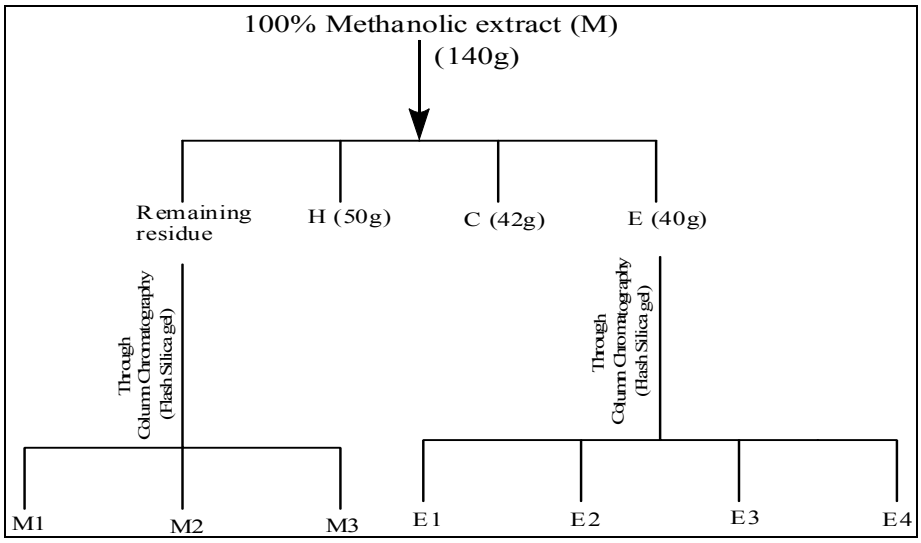

Fig. 1 Schematic diagram showing preparation of extracts and fractions of C.roseus.

M ; 100% methanol
M1 ; 80% methanol and 20% ethylacetate
M2 ; 60% methanol and 40% ethylacetate
M3 ; 40% methanol and 60% ethylacetate
E ; 100% ethylacetate
E1 ; 80% ethylacetate and 20% chloroform
E2 ; 60% ethylacetate and 40% chloroform
E3 ; 40% ethylacetate and 60% chloroform
E4 ; 20% ethylacetate and 80% chloroform
C ; 100% chloroform
H ; 100% n-hexane

After 5 min, 0.3 mL of 10% $AlCl_3$ was added; at 6min, 2mL of 1M NaOH was added. Water (2.4mL) was then added to the reaction flask and mixed well. Absorbance of the reaction mixture was measured at 510nm using a spectrophotometer (U-2800, Hitachi Instruments Inc., Tokyo, Japan). TFC amounts were determined as catechin equivalents (g/100g of dry plant matter). All samples were analyzed thrice and results averaged.

DPPH free radical scavenging assay

The DPPH assay was performed to determine the free radical scavenging activities of crude extracts as described by Bozin et al [(1)]. The samples (from 0.2 to 500µg/mL) were mixed with 1mL of 90 µM DPPH solution and filled up with 95% Methanol, to a final volume of 4mL. The absorbance of the resulting solutions and the blank were recorded after 1h at room temperature. Butylated hydroxytoluene (BHT) was used as a positive control. For each sample, three replicates were recorded. The disappearance of DPPH was read spectrophotometrically at 515nm using a spectrophotometer (U-2800, Hitachi Instruments Inc., Tokyo, Japan). Inhibition of free radical by DPPH in percent (%) was calculated in the following way:

$$\%I = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction mixture excluding the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} values, which represented the concentration of antioxidants that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

Antioxidant activity determination in linoleic acid system

The antioxidant activity of crude extracts determined in terms of measurement of % inhibition of peroxidation in linoleic acid system [7]. 5 mg extracts of each treatment were added to a solution mixture of 13 ml of linoleic acid, 10ml of 99.8% ethanol and 10 ml of 0.2 M sodium phosphate buffer of pH 7.The mixture was diluted to 25 ml with distilled water. The solution was then incubated at 40^{o}C and degree of oxidation was measured using thiocyanate method [17] with 10ml of 75% ethanol, 0.2 ml of

an aqueous solution of 30% ammonium thiocyanate, 0.2 ml of sample solution and 0.2 ml of ferrous chloride ($FeCl_2$) solution (20mM in 3.5% HCl) being added sequentially. After 3 min of stirring, the absorption values of mixures were measured at 500nm. These values were taken as peroxide contents.

A control with linoleic acid without extracts was performed. Synthetic antioxidants; butylated hydroxytoluene (BHT) and ascorbic acid (200 ppm) were used as positive control. The maximum peroxidation level observed as 360 h (15 days) in the sample that contained no oxidation component was used as a test point. Percent inhibition of linoleic acid peroxidation was calculated in the following way:%I = 100 – [(Abs. increase of sample at 360 h/ Abs. increase of control at 360 h) × 100]

Statistical analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of difference $p \leq 0.05$ was considered to denote a statistically significance All data were presented as mean values ± standard deviation (SD).

Results and discussion

Total Phenolic and total flavonoid contents (TPC)

We determined the total phenolic contents (TPC) and total flavonoid contents (TFC) in different solvent extracts and fractions of *C. roseus* shoots as shown in Fig. 2 and 3. The amounts of TPC and TFC extracted from *C. roseus* shoots in different solvent systems were in the ranges 3.2 to 8.5 GAE (g/100g per dry matter) and 1.8 to 5.4 CE (g/100g per dry matter) respectively. 100% methanolic extract of C.roseus showed the highest TPC and TFC, 8.5 and 19.8 g/100 g, respectively. These differences in the amount of TPC and TFC may be due to varied efficiency of the extracting solvents to dissolve endogenous compounds. The ability of different solvents to extract TPC and TFC was of the order: M> E> M1> M2> M3> E2> E1> E3> E4> C> H. 100% Methanol extracted maximum amount of antioxidant compounds while n-hexane being non polar in nature was the least effective for extraction of phenolics and flavonoids. Methanol is efficient and widely used to extract antioxidative components including phenolic acids [14]. Sultana et al [15] extracted antioxidative compounds from corncob and found that methanol gave maximum and n-hexane gave minimum antioxidant yield. Similar results were observed in the present investigations as methanol was most effective and n-hexane was least effective to extract antioxidative compounds.

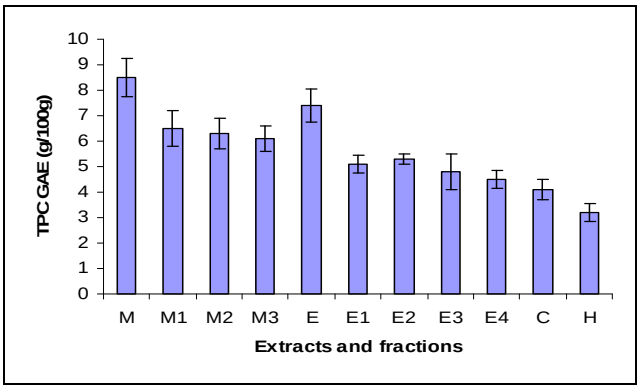

Fig.2 Total Phenolic Contents GAE (g/100g of per dry matter) of different extracts and fractions of *C. roseus* shoots. Values are mean ± SD of samples analyzed in triplicate. All calculations made on dry basis.

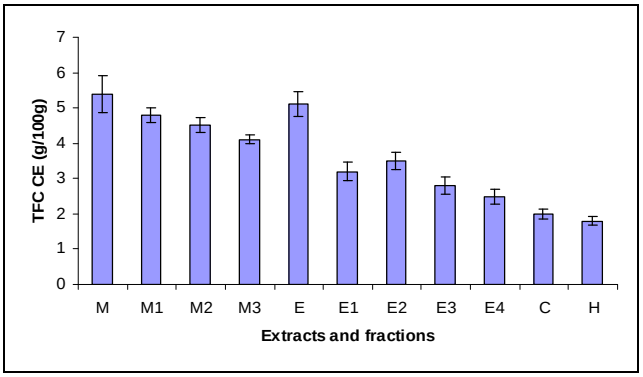

Fig.3 Total flavonoid Contents CE (g/100g of per dry matter) of different extracts and fractions of *C. roseus*. Values are mean ± SD of samples analyzed in triplicate. All calculations made on dry basis.

DPPH radical scavenging assay

We investigated the free radical scavenging activity of extracts and fractions of *C. roseus* shoots. Free radical scavenging activities of extracts and fractions of *C. roseus* shoots were measured by DPPH assay. Extracts and fractions of *C. roseus* shoots showed excellent radical scavenging activity, with IC_{50} (the extract concentration providing 50% of inhibition) values ranging from 28.2 to 119 µg/ml as shown in Fig 4 The free radical scavenging activity of 100% methanolic extract was superior to that of other extracts and fractions. While 100% methanolic extract exhibited lowest IC_{50} value and n-hexane showed highest IC_{50} value it means antioxidant activity of methanolic extract was higher and lowest of n-hexane. When compared with the synthetic antioxidant BHT, all extracts and fractions offered lower antioxidant activity. The order of IC_{50} value offered by Extracts and fractions of *C. roseus* shoots were as follows: BHT< M< E< M1< M2< M3< E2 <E1< E3< E4< C< H. To best of our knowledge no earlier reports are available regarding the DPPH radical scavenging activity of C.roseus shoots with which to compare the results of our present analysis.

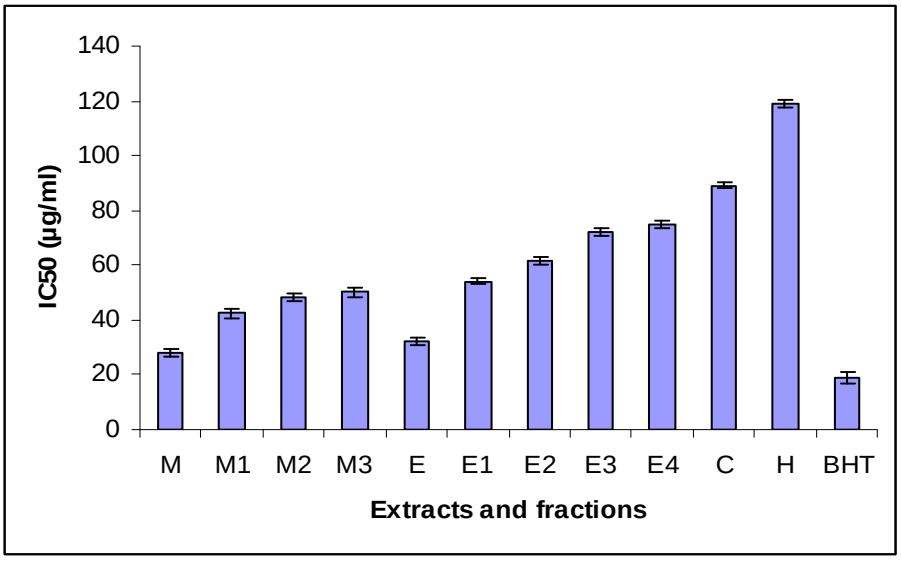

Fig. 4 Antioxidant activity of different solvents extract and fractions of *C. roseus* determined as DPPH radical scavenging (IC_{50}ug/ml). Values are mean ± SD of samples analyzed in triplicate. All calculations made on dry basis.

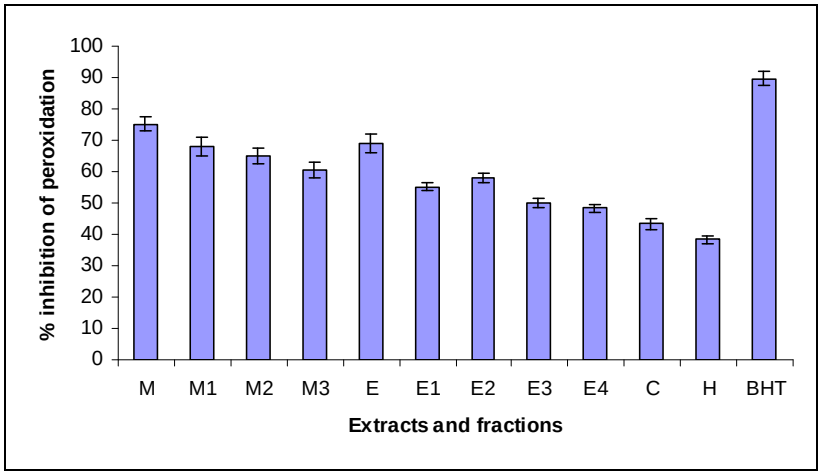

Fig.5 Antioxidant activity of different solvents extract and fractions of C .roseus determined as percent inhibition of linoleic acid oxidation. Values are mean ± SD of samples analyzed in triplicate. All calculations made on dry basis.

Percentage inhibition of linoleic acid oxidation.

We determined the percentage inhibition of linoleic acid oxidation as exhibited by the shoots of C.roseus extracts and fractions as shown in Fig 5. 100% methanol extract offered significantly ($p < 0.05$) higher inhibition of peroxidation (75.1%) other extracts and fractions showed lowest inhibition then methanolic extract. When the inhibitions of linoleic acid oxidation of C.roseus shoots extracts and fractions were compared with BHT, all the extracts and fractions exhibited significantly ($p < 0.05$) lower antioxidant activity than that shown by BHT (89.7%). The order of inhibition of linoleic acid oxidation offered by extracts and fractions of C.roseus shoots were as follows: BHT> M> E> M1> M2> M3> E2> E1> E3 > E4> C> H. Due to lack of data on the percentage inhibition of linoleic acid oxidation of C.roseus shoots extracts and fractions we could not compare the results of our present study with the literature.

Conclusion

In this study we presentd results of various in vitro antioxidant assays to investigate the effect of solvent on the extraction and total antiradical potential of different extracts of *Catharanthus roseus*. The various antioxidant assays of plant extract and its fractions revealed *C. roseus* to be a good source of natural antioxidants. 100% methanolic extract and 100% ethylacetate fraction of *C. roseus* shoots were found to exhibit highest antioxidant activity. On the basis of results of this study it is clear that *C. roseus* shoots extract can be used as a potential source of easily accessible natural antioxidants as well as in pharmaceutical applications.

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