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Antioxidant and antihemolytic activities of common Nilgiri barberry (*Berberis tinctoria* Lesch.) from south India

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Abstract

In this study, the potential of Common *Nilgiri Barberry* (*Berberis tinctoria* Lesch.) fruits as nutraceutical/functional food, polyphenolic contents and the *in vitro* antioxidant and antihemolytic activity were determined. The phenolic content (TP) of the fresh fruits is 410 ± 0.082 mg gallic acid equivalents (GAE)/100g and total flavonoid content (TF) is 320 ± 0.120 mg quercetin equivalents (QE)/100g. The methanol extract exhibited scavenging capacity towards 1, 1 – diphenyl – 2- picryl hydrozyl (DPPH·), superoxide anion, hydroxyl ion radicals and nitric oxide. The TEAC of fruit extract ranged from 1.063-2.364 mM TE/g. The extract also exerted strong reducing capacity and had strong Fe^{3+} chelation (EC_{50} 45.24 ±1.42 µg mL^{-1}), and remarkable reduction of erythrocyte hemolysis (EC_{50} 71.1 ± 0.22 µg ml^{-1}). Positive correlations were observed between polyphenolic contents and the antioxidant capacities. In conclusion, the Barberry fruit from India, endowed with polyphenols, could be a potential source of for the development of natural antioxidants/nutraceuticals.

Introduction

Over the past few decades, increasing epidemiological studies and intervention trails have consistently reiterated the role of consumption of fruits and vegetables as antioxidants in the prevention of the degenerative diseases (Halliwell *et al*., 1997; Lieu, 2003). The protective effects of fruits are mostly related to the antioxidant components i.e. vitamins, flavonoids, and carotenoids (Pietta, 2000; Rice-Evans, 2001). To derive the maximum health benefits, sufficient amounts of phytochemicals from a variety of plant sources as fruits, vegetables and whole grains based foods are recommended (Prior, 2003). Polyphenols in fruits scavenge active oxygen species and effectively prevent oxidative cell damage. Previous researches have demonstrated that the phenolic compounds occurring in the fruits possess antioxidant activities and health benefits (Kahkonen *et al*., 2001; Garcia-Alonso *et al*., 2004; Sreeramulu *et al*., 2010). Therefore, it is of great interest in research concerning the antioxidant ability of fruits which would be used as nutraceuticals/functional food.

Berberis tinctoria Lesch., belonging to the family Berberidaceae, is commonly known as common *Nilgiri Barberry* (Locally '*Oosikala* or '*Jakkala Hannu*') and is found only in Inner Shola forest of the Nilgris and in Palni hills, India at an altitude of 1,800 m (Wealth of India, 1988). Earlier, hepatoprotective activity and antioxidant activity of the leaves of *B. tinctoria* were evaluated (Murugesh *et al*., 2005). Sasikumar *et al*. (2007) reported antibacterial activity of various solvent extracts of *B. tinctoria* root. Methanol extracts of root and root bark of *B. tinctoria* were found to possess potential hydrogen donating on DPPH and strong reductive capacity (Sasikumar *et al*., 2009). The alkaloid, berberine isolated from *Berberis* species is reported for various infectious diseases ans possesses antimicrobial activity (Wealth of India, 1988; Singh, 2009).

As far as our literature survey could ascertain, no information was available on *in vitro* antioxidant properties and polyphenolic contents of underexploited wild edible fruits from Nilgiri District, Western Ghats, India. Hence, in order to supply more scientific evidence for research and development of underexploited fruits, we aimed to study the total phenolic and total flavonoid contents, and the antioxidant capacities of Barberry fruit.

Materials and Methods

Chemicals

Gallic acid was purchased from Riedel-de-

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Hahn, Germany. Foli-Ciocalteu phenol reagent, 2-deoxy-D-ribose, potassium ferricyanide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), L-ascorbic acid, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS), quercetin, (+)-catechin, 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), nicotinamide adenine dinucleotide (NADH), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), ethylene diamine tetraacetic acid disodium salt (EDTA-Na_2), hydrogen peroxide, trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Merck Co., India. All other chemicals and solvents used were of analytical grade.

Preparation of Barberry extract (BE)

The fully ripened fruits of *B. tinctoria* were collected from Doddabetta forest range, the Nilgiris, Tamil Nadu during May 2007 and June 2007. The fruits were pooled and were kept in cold (-4^{0}C) dark storage until processed. The voucher specimen was prepared and deposited at the herbarium of Karpagam University, Coimbatore. Briefly, the frozen berries (100 g) were blended, exhaustively extracted with 500 mL of methanol (1:5 v/v) and centrifuged (3000 g, Remi, India) for 15 min at 4^{0}C. The process was repeated thrice. The supernatants were then combined and filtered over Whatman No. 1 filter paper. The filtered extract was concentrated at 4^0 ± 1^{0}C by rotary flash evaporator (Buchi type rotavapor) under reduced pressure. The resultant crude extract was used to analyse the polyphenolic contents and to determine *in vitro* antioxidant properties.

Total phenolics and flavonoid determination

Total soluble phenolics content of Barberry fruit was measured according to the Folin-Ciocalteu method as previously described with minor modifications (Singleton *et al*., 1997). The absorbance was measured at 650 nm in UV-Vis spectrophotometer (Shimadzu, Japan). The TP was expressed as gallic acid equivalent (mg GAE/100g fresh). The content of total flavonoid in *B. tinctoria* fruit was based on aluminium chloride ($AlCl_3$) method described previously (Ordonez *et al*. 2006). The absorbance was measured at 420 nm. The total flavonoid was calculated as quercetin equivalent (mg QE/100g fresh).

DPPH· radical scavenging activity

The determination of DPPH· stable radical scavenging activity was based on the method described by Singh *et al*. (2002). Briefly, one millilitre of aliquots of the BE and standards (5 -1000 µg mL-1) was added to MeOH solution of DPPH· (5 mL, 0.1 mM) and vortexed. After 20 min reaction at 25^{0}C, the absorbance was measured at 517 nm against a blank in a UV-VIS spectrophotometer (Shimadzu, Japan). BHT was used for comparison. The percentage quenching of DPPH· was calculated as follows:

% inhibition of DPPH·=1-Sample517nm/ Control517nmx100

where, Sample517nm was absorbance of the sample and Control517nm was absorbance of control.

Reductive capacity

The reducing power of the fruit extract was measured using potassium ferricyanide reduction method (Oyaizu, 2006). Various concentrations of BE and standards (50-1000 µg mL^{-1}) were added to 2.5 mL of (0.2 M) sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe_3(CN)6$] (1%) solution and vortexed. After incubation at 50 ^{0}C for 20 min, 2.5 ml of TCA (10%) was added to all the tubes, centrifuged (3000 x g) for 10 min and 5 mL of the supernatant was mixed with 5 mL of deionized water. To this, one millilitre of $FeCl_3$ (1%) was added to each test tube and incubated at 35 ^{0}C for 10 min. The formation of Perl's Prussian colour was measured at 700 nm in a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). Increased absorbance of the reaction mixture indicated increasing reducing power. BHT and BHA were used for comparison.

O^{2-} scavenging activity

Super oxide anion radicals generated in the phenazine methosulfate-reduced form of nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by the reduction of NBT (Yu, 2006). Superoxide radicals were generated in 1.25 mL of tris–HCL buffer (16 Mm, pH 8.0), 0.25 mL of NBT (150 µM), 0.25 mL of NADH (468 µM) and different concentrations (50-1000 µg mL^{-1}) of BE. The reaction was started by adding 0.25 mL of PMS (60 µM, PMS in 100 mM PB; pH 7.4) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm. BHT was used for comparison.

OH^- scavenging activity

Hydroxyl radicals (OH^-) were generated by a fenton reaction system, and the scavenging capacity towards the OH^- radical was measured by using a deoxyribose method (Halliwell *et al*., 1997). Different concentrations of the BE (50-1000 µg mL^{-1}) were added with phosphate buffer (50 mM; pH 7) to

the final volume of 1 mL. Then, 0.2 mL of EDTA (1.04 mM), 0.2 mL of $FeCl_3$ (1.0 mM) and 0.2 mL of 2-deoxyribose (60 mM) were added. The mixture was kept in a water bath at 37 °C for 1 hour. After incubation, 2 mL of cold TBA (10 g/l) and 2 mL of HCl (25%) were added to the reaction mixture. The mixture was incubated at 100°C for 15 min. After cooling, the absorbance of the solution was read at 532 nm. BHT was used for comparison.

Nitric oxide radical inhibition activity

Nitric oxide (NO) generated from sodium nitroprusside (SNP) in aqueous solution at physiological was estimated by the use of Griess reaction (Green *et al*., 1982). The reaction mixture (3 mL) containing SNP (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and BE at different concentrations (50-1000 µg mL^{-1}) were incubated at 25 ºC for 150 min. After incubation, 0.5 mL of the reaction mixture was pipetted, mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25ºC. The absorbance of pink coloured chromophore formed was measured at 540 nm. BHT was used for comparison.

Fe^{3+} chelation assay

The Fe^{3+} chelating activity was determined by the method described by Singh and Rajini (2004). Briefly, different concentrations of BE were mixed with 1 mL of $FeSO_4$ (2 mM) and ferrozine (5 mM). The mixture was made into 4 mL with deionized water. Absorbance was measured at 562 nm after 10 min. EDTA-Na_2 and was used for comparison.

Trolox equivalent antioxidant capacity (TEAC)

The ABTS•+ radical cation was produced by oxidising ABTS•+ with potassium persulfate ($K_2S_4O_8$) with minor changes (Re *et al*. 1999). The ABTS•+ solution (7 mM) was oxidized with $K_2S_4O_8$ (2.4 mM) for 12 h at room temperature in the dark. The ABTS•+ solution was then diluted by mixing 990 µl ABTS•+ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 at 734 nm. Fruit extract and BHT of various concentrations (20-100 µg mL^{-1}) were allowed to react with 1.0 ml of the ABTS•+ solution and the absorbance was measured at 734 nm after 7 min. Standard curves of the reaction of Trolox with ABTS and the readings were used for measurements of TEAC of the extract tested, expressed in TEAC in mM of Trolox (TE)/g dry material of sample.

Inhibitory effects on H_20_2 induced erythrocyte hemolysis

The antihemolytic activity of fruit extract was examined by the method described previously (Naim, 1977). The erythrocytes from cow blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4), until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of sample (200-1000 µg mL^{-1}) with saline or buffer were added to 2 mL of the suspension of erythrocytes and the volume was made up to 3.5 mL with saline or buffer. This mixture was preincubated for 5 min and then 0.5 mL H_20_2 solutions of appropriate concentration in saline or buffer were added. The concentration of H_20_2 in the reaction mixture was adjusted so as to bring about 90% hemolysis of blood cells after 120 min incubation. Incubation was concluded after these time intervals by centrifugation at l000 g for during 5 min. The extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation.

Statistical analysis

The results of this investigation are the mean ± standard deviation of three parallel measurements. Linear regression analysis was used to calculate the efficient concentration (EC_{50}) values.

Results and Discussions

Determination of total phenolics and flavonoid contents

The phenolic concentration was 410 ± 0.02 mg/100 g of gallic acid equivalents (GAE) of fresh mass with reference to a standard curve ($R^2 = 0.9968$). Our data revealed that the TPC in barberry fruit was high when compared to other commonly consumed fruits including plum (42-109 mg/100 g) and peaches (21 - 111 mg/100 g) (Gil *et al*., 2002). The flavonoid content was 320 mg of quercetin equivalents (QE) in 100 g of fresh mass with reference to a standard cure ($R^2 = 0.9665$). The result is close to Gala apple peel (309 ± 0.5 mg GAE/100 g FW) (Hassimotto *et al*., 2005).

DPPH· quenching activity

With regard to DPPH· stable scavenging activity or H-donor activity of BE, a dose dependent inhibition was observed (Table 1). The BE exhibited impressive DPPH· scavenger activity with 90.63 ± 0.26 % at 1000 µg mL^{-1} concentration. On the other hand, BHT was able to scavenge 88.87 % at 1000

Table 1. Antioxidant capacities of methanol extract of *B. tinctoria* fruit and BHT

Concentration of extract and standard (µg/ml)	Percentage activity (%)			
	DPPH	$O_2^{\cdot-}$	OH·	NO·
50	55.65 ± 1.33[1] (55.30 ± 0.89)[2]	40.08 ± 1.32 (52.47 ± 0.84)	64.57 ± 0.82 (42.71 ± 0.52)	44.97 ± 1.02 (47.62 ± 0.94)
100	59.04 ± 0.84 (57.88 ± 1.26)	43.15 ± 0.78 (61.47 ± 1.09)	68.98 ± 0.33 (55.38 ± 0.99)	50.45 ± 0.64 (69.40 ± 1.36)
250	71.50 ± 1.52 (65.96 ± 0.78)	52.89 ± 0.59 (75.45 ± 1.63)	74.87 ± 1.44 (62.91 ± 0.62)	54.67 ± 1.15 (74.90 ± 0.93)
500	78.34 ± 0.34 (69.06 ± 1.05)	55.42 ± 0.84 (83.62 ± 1.43)	80.98 ± 1.53 (76.14 ± 0.76)	60.32 ± 1.61 (84.38 ± 1.24)
750	88.81 ± 0.83 (77.86 ± 1.47)	63.18 ± 0.67 (88.28 ± 0.68)	86.08 ± 0.48 (84.37 ± 0.97)	67.48 ± 0.76 (89.62 ± 0.39)
1000	90.63 ± 0.26 (88.87 ± 0.93)	66.24 ± 0.44 (94.19 ± 0.74)	89.88 ± 0.25 (88.41 ± 0.49)	72.89 ± 0.23 (93.64 ± 0.57)
EC_{50} values (µg mL^{-1})				
Barberry	9.85 ± 1.33	64.65 ± 0.82	79.98 ± 1.02	75.21 ± 1.32
BHT	26.12 ± 0.84	16.05 ± 0.33	16.44 ± 0.64	26.12 ± 0.84

[1]The data are presented as mean value ± standard deviation SD (n = 3)
[2]Values given in the parentheses are percentage activity of standard, BHT

µg mL^{-1}. Based upon the measured EC_{50} values (Table 2), the DPPH· quenching ability (9.85 ± 1.33 µg mL^{-1}) was significantly more efficient than BHT (26.12 ± 0.04 µg mL^{-1}). DPPH· scavenging activity was also more pronounced than that of commonly consumed fruits including Syzygium cumini (IC_{50} 168 µg/ml) (Banerjee *et al*., 2005) and other fruits commonly consumed in India (Vijaya Kumar Reddy *et al*., 2010). DPPH· scavenging of BE extract was linearly correlated with TPh (r^2 = 0.965) and TFl contents (r^2 = 0.957).

Reducing capacity

The reduction of ferrous ion (Fe^{3+}) to ferric ion (Fe^{2+}) is measured by the intensity of the resultant Persian-blue solution which absorbs at 700 nm. In the assay, the barberry extract was able to convert the oxidized form of Fe^{3+} into Fe^{2+} (Figure 1). The reducing capacity (RC) of the methanol extract, with OD value of 0.754 ± 0.021, was significantly lesser than that of BHT (1.022 ± 0.42) at 70 µg mL^{-1}. The reducing properties are generally associated with the presence of reductones. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Gordon, 1990). The result indicated that the marked reducing power of the fruit extract seems to be due to presence of polyphenols which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them into more stable products and terminate free radical chain reaction. Significant correlations were observed between RC and, TPl (r^2 = 0.993) and TFl (r^2 = 0.993).

$O^{2\cdot-}$ scavenging activity

The $O^{2\cdot-}$ radical is one of the most dangerous free radicals in humans (Schlesier *et al*., 2002) and also the source of hydroxyl radical (OH·). In the PMS/NADH-NBT system, The $O^{2\cdot-}$ radicals derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. In the present work, the dose dependent inhibition of $O^{2\cdot-}$ generation by BE is illustrated in Figure 2C. The extract exhibited 66.24 ± 0.44 % of $O^{2\cdot-}$ scavenging at the concentration of 1000 µg mL^{-1} with an EC_{50} value of 64.65 ± 0.82 µg mL^{-1}. As reported in Table 1, the BE exerted noticeable scavenging effect on $O^{2\cdot-}$ radicals though the activity was significantly lower than the BHT. The present finding is more efficient when compared with the values (EC_{50} 260 µg/ml) obtained from methanol extract of *Syzygium cumini* (Banerjee *et al*., 2005). The $O^{2\cdot-}$ scavenging and was in well correspondence with TPh (r^2 = 0.994) and TFl (r^2 = 0.994).

OH· scavenging activity

Hydroxyl radical (OH·), is the most reactive free radical, has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity (Naidu *et al*., 2008). As illustrated in Table 1, the BE inhibited the degradation of 2-deoxyribose in a dose dependent manner. Its ·OH scavenging activity was 89.88 ± 0.25% at the concentration of 1000 µg mL^{-1}. However, this value was significantly lower than the values of positive control BHT (88.41% at 1000 µg mL^{-1}). The EC_{50} value of BE (79.98 ± 1.02 µg mL^{-1}) was significantly lower when compared to BHT (16.44 + 0.04 µg mL^{-1}) (Table 1). The ·OH scavenging ability of BE extract was also more efficient as compared with commonly consumed fruit in India, *S. cumini* (EC_{50} 428 µg/ml) (Banerjee *et al*., 2005). A positive correlation was observed between OH scavenging and TPl (r^2 = 0.976) and TFl (r^2 = 0.94).

NO scavenging activity

The NO generated from SNP at physiological pH reacts with oxygen (O_2) to form nitrite ions. From the results obtained, it was found that the barberry fruit exerted dose dependent inhibitory potential

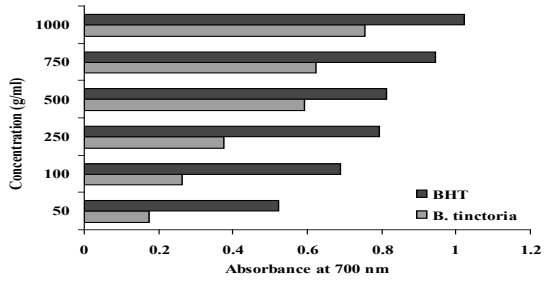

Figure 1. Reducing capacity (RC) of methanol extract of *B. tinctoria* fruits and butyl hydroxyl toluene. Data are mean ± SD (n = 3)

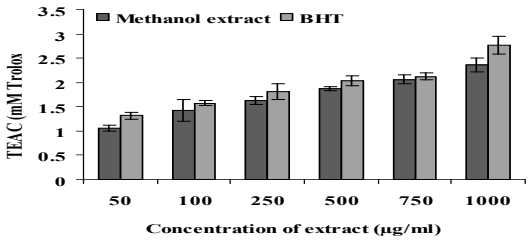

Figure 2. Trolox Equivalent Antioxidant Capacity of (TEAC) Barberry extract and BHT. Data are mean ± SD (n = 3)

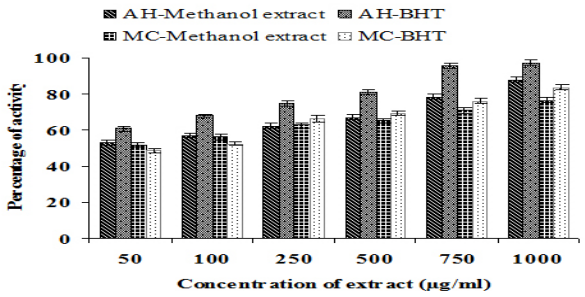

Figure 3. Iron chelating capacity and Inhibition of erythrocyte hemolysis by Barberry extract and BHT (butyl hydroxyl toluene). AH – antihemolytic activity, MC – metal chelating activity. Data are mean ± SD (n = 3)

towards nitric oxide with an EC_{50} value 75.21 ± 1.32 µg mL^{-1} (Table 1). The NO scavenging activity may be due to the antioxidant components in the barberry extract which compete with O_2 to react with NO (Marcocci et al., 1958). The NO scavenging was in well correspondence with TPh (r^2 = 0.963) and TFl (r^2 = 0.976).

TEAC

The decolourization of ABTS.+ cation also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate these radical species (Pellegrini *et al*., 1999). In the present study, the extent of inhibition of the absorbance of the ABTS is plotted as a function of concentration in order to determine the TEAC. The TEAC of fruit extract ranged from 1.063-2.364 mM TE/g whilst the TEAC of BHT from 1.313-2.765 mM TE/g (Figure 2). The ABTS scavenging activity of the fruit extract was comparable to that of BHT.

Fe^{3+} chelation

Metal chelating activity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. In this study, chelating activity was assayed by the inhibition of formation of red-coloured ferrozine and ferrous complex. The positive control in this assay EDTA exerted the strongest chelating activity and at 1000 µg mL^{-1} there was a 96.08 ± 1.64% chelating effect (data not shown), which was significantly higher than that of BE. Iron chelating ability of EDTA was higher than that of phenolic compounds (Andjekovic *et al*., 2006). The EC_{50} value of fruit extract (45.24 ± 1.42 µg mL^{-1}) was significantly higher than that of BHT (46.34 ± 0.78 µg mL^{-1}). The data obtained from this assay revealed that the BE extract demonstrated as an effective capacity for metal-binding, suggesting that the raspberry extract may play a protective role against oxidative damage by sequestering Fe^{2+} ions.

Antihemolytic activity

Biomembranes may be most susceptible to free radical attack due to its content of polyunsaturated fatty acids (Sekiya *et al*., 2005). In this study, lipid oxidation of cow erythrocyte by H_2O_2 induced peroxy radicals leads to erythrocyte hemolysis. The fruit extract inhibited the hemolysis of cow erythrocyte in a dose dependent manner (Figure 3). The *in vitro* membrane stabilizing property of this fruit was observed by their ability to provide protection against H_2O_2 induced cow erythrocyte hemolysis. The EC_{50} value of the fruit extract, representing the concentration required to inhibit 50% of erythrocyte hemolysis, was 71.1 ± 0.22 µg ml^{-1}. The antihemolytic action of the fruit on cow erythrocytes is at least partly due to the antioxidative property of the phenols.

Recent years have seen an exponential increase in research antioxidant properties of fruits and vegetables. This investigation was performed to elucidate the nutraceutical potential and to develop products of added value of underutilized fruits of *B. tinctoria* which are potentially valuable dietary resource. On the basis of all reported findings, the Barberry displayed remarkable antioxidant activities as it was able to scavenge the ROS and reactive nitrogen species (RNS). The results obtained strongly point towards prospective antioxidant capacity of underexploited barberry fruits. In thus study, the TPI and TFI contents of *B. tinctoria* fruits were well correlated with all the tested assays. Many works have shown that there has been positive correlation between polyphenolic content and antioxidant activity (Connor *et al*., 2002; Sun and Ho, 2005). Therefore it was considered that the high antioxidant activity

of the fruit extract could be attributable to its high amount of polyphenolic content. Thus, the Nilgiri barberry could prevent the free radical mediated damage to biomolecules such as lipids, proteins and DNA.

Conclusions

In conclusion, barberry fruit extract efficiently scavenged DPPH, O^{2-}, NO, $OH^{\cdot}$ and $ABTS^{\cdot+}$ radicals, chelated ferrous ion and inhibited hemolysis *in vitro*. Obviously, this underexploited fruit may be used in the development of functional food and raw materials of medicine. We would emphasize that the present investigation relates only to *in vitro* studies, and *in vivo* or dietary intervention studies are warranted.

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