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Antioxidant Properties of Methanolic Extract of *Dolichos biflorus* (Linn.)

A. KOTTAI MUTHU*, S. SETHUPATHY†, R. MANAVALAN and P.K. KARAR
Department of Pharmacy, Annamalai University, Annamalai Nagar-608 002, India
Fax: (91)(4144)238080; Phone : 09443171712 (M)
E-mail: arthik03@yahoo.com

The present study was conducted to evaluate the *in vitro* and *in vivo* antioxidant properties of methanolic extract of *Dolichos biflorus* (Linn.). The *in vitro* antioxidant potential of the plant extract was evaluated using three separate methods namely inhibition of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), H_2O_2 assay and nitric oxide radical inhibition assay. Rabbits were fed high fat diet and the oxidative damage was explored by assessing parameters such as superoxide dismutase (SOD) gluthathione peroxidase (GP_x) and gluthathione-S-transferase (GST) in liver, heart and aorta. The effect of co-administration of two different doses (200mg/kg and 400 mg/kg body weight) of *Dolichos biflorus* extracts on the above parameters was also investigated and compared with standard drug atorvastatin. The *in vitro* antioxidant potential of methanolic extract of *Dolichos biflorus* when compared with the standard antioxidant like ascorbic acid, rutin, BHT exerted remarkable antioxidant activity. Antioxidant enzymes such as SOD, GP_x and GST too showed enhanced activities on co-administration of methanolic extract of *Dolichos biflorus*. Higher dose of the plant extract was found to be more effective than lower dose. It is concluded that administration of *Dolichos biflorus* manifests a protective action against HFD induced oxidative stress in different tissues in rabbits and exhibits stronger antioxidant activity in *in vitro* studies.

Key Words: Methanolic extract, *Dolichos biflorus*, Antioxidant, Rabbits.

INTRODUCTION

Reactive oxygen species in biological systems are related to free radicals, known to interact with many cellular components and be involved in a variety of physiopathological process[1]. Free radical often attacks DNA, protein molecules, enzymes and cells leading to alterations in genetic material and cell proliferation.

In order to enhance cellular protection against free radical injury, the antioxidants of enzymatic (superoxide dismutase, peroxidase, catalase,

†Division of Biochemistry, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar-608 002, India

gluthathione-S-transferase, *etc.*) and non-enzymatic (vitamin C, vitamin E, β-carotene, *etc.*) may play an important role as free radical scavengers.

The use of spices and herbs as antioxidants in processed foods is a promising alternative to the use of synthetic antioxidants like butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), ascorbic acid, curcumin, *etc*. The reasons for increasing interest in natural antioxidants are: doubts on the safety of use of synthetic substances, presence of various phytochemicals that can affect the aetiology and pathology of chronic diseases, the ageing process[2]. Hyperlipidemia is an important risk factor in the initiation and progression of atherosclerosis and coronary heart disease. Oxidative stress may play a key role in initiation of atherosclerosis. The plant *Dolichos biflorus* Linn, (Fabaceae), commonly known as kollu in Tamil and horse gram in English is chosen in this investigation. Branches of *D.biflorus* are suberect or twining, glabrescent, leaflets are 2.5 to 5 cm, broardly lanceolate, stipules subulate, seeds 5-6, compressed reniform, grey or reddish brown in colour[3]. Dolichos biflorus has antitumor activity[4], the root of the plant is used as expectorant in China[5], the entire dried plant is used in abortion in India[6] and it is also used in menstrual problems[7].

EXPERIMENTAL

Whole plants of *D.biflorus* were collected from Sankaran coil, Tirunelveli district of Tamilnadu, India. Taxonomic identification was made from Botanical Survey of Medicinal Plant Unit, Siddha, Government of India, Palayamkottai, Tamilnadu. Four months old whole plants were dried under shade, segregated and pulverized by a mechanical grinder and passed through a 40 mesh sieve. The powdered materials were successively extracted by hot continuous percolation method[8] in soxhlet apparatus. The extracts were suspended[9] in 2% tween 80.

Animals and treatment for *in vivo* study

New Zealand White rabbits of weighing 900-1100 g were procured from the Central Animal House, Rajah Muthiah Medical College, Annamalai University. The animals were kept in cages, 2 per cage, with 12 : 12 h light and dark cycle at 25 ± 2ºC.The animals were maintained on their respective diets and water *ad libitum*. Animal were divided into following five groups of six animals each:

Group I (Control) : Standard chow diet
Group II : High fat diet
Group III : High fat diet plus methanolic extract of *D. biflorus* (dose-I 200mg/kg body weight/day)
Group IV : High fat diet plus methanolic extract of *D.biflorus* (dose-II 400mg/kg body weight/day)

Group V : High fat diet plus standard drug atorvastatin (1.2 mg/kg body weight/day)

Animal diet

The compositions of the two diets were as follows:

Control diet: Wheat flour 22.5 %, roasted Bengal gram powder 60 %, skimmed milk powder 5 %, casein 4 %, refined oil 4 %, salt mixture with starch 4 % and vitamin and choline mixture 0.5 %.

High fat diet: Wheat flour 20.5 %, roasted Bengal gram 52.6 %, skimmed milk powder 5 %, casein 4 %, refined oil 4 %, coconut oil 9 %, salt mixture with starch 4 % and vitamin and choline mixture 0.5 % and cholesterol 0.4 %.

***in vivo* Antioxidant study**

Superoxide dismutase (SOD) assay[10]: 0.5 mL of sample (tissue homogenate) was diluted to 1 mL with ice cold water. 2.4 mL of ethanol and 1.5 mL chloroform (in chilled condition) were added. This mixture was shaken for 1 min at 4ºC and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 mL sodium pyrophosphate buffer, 0.1 mL phenazine methosulphate, 0.3 mL nitroblue tetrazolium, appropriately diluted enzyme preparation and water in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30ºC for 90 s, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was allowed to stand for 10 min and then centrifuged. The colour intensity of the chromophore in butanol layer was measured at 560 nm against butanol blank and a system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one min under the assay conditions and the activity was expressed as units/mg protein.

Glutathione peroxidase (GP_x) assay[11]: To 0.2 mL of tris buffer, 0.2 mL EDTA, 0.1 mL sodium azide, 0.5 mL sample (tissue homogenate) and 0.2 mL GSH were added followed by 0.1 mL H_2O_2. The contents were mixed well and incubated at 37ºC for 10 min along with a tube containing all the regents except the sample. After 10 min, the reaction was arrested by the addition of 0.5 mL of 10% TCA, centrifuged and the supernatant was assayed for GSH.

Glutathione S-transferase (GST) Assay[12]: 1.0 mL phosphate buffer, 0.1 mL 1-chloro-2,4-dinitro benzene (CDNB) and 0.1 mL tissue homogenate were taken and the volume was adjusted to 2.9 mL with distilled water. The reaction mixture was pre-incubated at 37ºC for 5 min and it was started by the addition of 0.1 mL 30 mM glutathione. The absorbance was

followed for 5 min at 340 nm. Reaction mixture without the enzyme was used as blank.

GST-activity was expressed as mM of CDNB-GSH conjugate formed/min/mg protein which was calculated using the formula,

$$\frac{\text{Optical density} \times 3 \times 1000}{9.6 \times 5 \times \text{ protein in mg}}$$

9.6 is the difference in the mm extinction coefficient between CDNB-GSH conjugate and CDNB.

***in vitro* Antioxidant study**

DPPH assay[13]: The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple-coloured methanol solution of DPPH. This spectrophotometric assay uses the stable radical 2.2'-diphenylpicrylhydrazyl (DPPH) as a reagent. Various concentration of the extracts (50 µL) in methanol were added to 5 mL of a 0.004 % methanol solution of DPPH. After a 0.5 h incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical, DPPH, in percent (1 %) was calculated in following way:

$$1\ \% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50 % inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate.

Hydrogen peroxide assay: A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 mL of the extract or standards in methanol were added to 2 mL of hydrogen peroxide solutions in PBS. The absorbance was measured at 230 nm after 10 min against a blank solution that contained extracts in PBS without hydrogen peroxide.

Nitric oxide radical inhibition assay[14]: Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modifed Griess Llosvay reaction[15]. In the present investigation, Griess llosvay reagent is modified by using naphthyl ethylene diamine dihydrochloride (0.1 % w/v) instead of 1-napthylamine (5 %). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and extract or standard solution (0.5 mL) was incubated at 25ºC for 2.5 h. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted and

mixed with 1 mL of sulphanilic 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 0.5 h at 25ºC. A pink coloured chrmophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions in micro titer plates using ELISA reader. IC_{50} value is the concentration of sample required to inhibit 50 % of nitric oxide radical.

Statistical analysis

Results were expressed as mean ± SE of six rabbits in each group. Two ways analysis of variance (Anova) with Scheffe's multiple comparisons test were used to determine the statistical significance. Significance level was fixed at 0.05.

RESULTS AND DISCUSSION

The *in vitro* antioxidant activities of the methanolic extract of *D. biflorus* studied were determined by three methods namely DPPH assay, H_2O_2 assay and nitric oxide free radical scavenging capacity are given Table-1.

TABLE-1
FREE RADICAL SCAVENGING CAPACITIES OF THE METHANOLIC EXTRACTS OF *D. biflorus* IN *in vitro* METHODS

Samples	DPPH assay (μg/mL) IC_{50} value ± SE*	H_2O_2 assay IC_{50} value ± SE*	Nitric oxide radical Inhibition assay IC_{50} value ± SE*
D. biflorus (methanolic extract)	63.1 ± 2.10	90.50 ± 3.70	115.5 ± 7.0
BHT	20.5 ± 0.50	25.75 ± 0.20	–
Ascorbic acid	4.5 ± 0.40	–	–
Rutin	43.6 ± 1.80	48.50 ± 0.16	106.3 ± 20.85

*Results are means of three experiments.

Free radical scavenging activity of the methanolic extract of *Dolichos biflorus* is concentration dependent and lower IC_{50} value reflects better protective action. The methanolic extract was able to reduce the stable free radical 2,2 diphenyl-1-picryl hydrazyl (DPPH) to yellow coloured diphenylpicryhydrazine with an IC_{50} of 60 μg/mL, exhibiting better activity than the synthetic antioxidant agent BHT IC_{50} of 20.5 μg/mL.

Antioxidant activity of methanolic extract of *Dolichos biflorus* by H_2O_2 scavenging method of assay and nitric oxide free radical inhibition assay shown better antioxidant activity as their IC_{50} values were found to be 90.5 μg/mL, 115.5 mg/mL than the IC_{50} values shown by synthetic antioxidant rutin 48.5mg/mL, 106.3 mg/mL.

The key role of phenolic compounds, flavonoids and terpinene as scavengers of free radicals is emphasized in several reports[16]. Antioxidant properties of the various extracts from many plants are of great interest in food industry since the possible use of natural additives emerged from a growing tendency to replace synthetic antioxidant by natural one[17].

The activities of SOD in the tissue like liver, heart and aorta were significantly lowered in rabbits fed with high fat diet than control group animals. After administration of methanolic extract of *Dolichos biflorus* doses (200 mg/kg body weight and 400 mg/kg body weight) along with HFD significantly increases the activities of SOD in tissues of rabbits.

The high fat diet can cause the formation of toxic intermediates in the intestine that can inhibit the activity of antioxidant enzyme[18]. The phytoconstituent of *Dolichos biflorus* extract increases the activity of SOD due to removed intestinal microflora in the presence of HFD, that could inhibit the enzyme activities.

The activities of glutathione peroxidase and glutathione-S-transferase results are shown in Tables 2 and 3, respectively. The activities of glutathione peroxidase and glutathione-S-transferase were also significantly decreased in tissues of rabbits fed with HFD as compared to the control rabbits. Administration of methanolic extract of *D. biflorus* (dose 200, 400 mg/kg) along with the HFD increased the activities of glutathione peroxidase and glutathaione-S-transferase in all the tissues as compared with HFD. A standard drug atorvastatin administered rabbits also showed elevated level of glutathione peroxidase and glutathione-S-transferase.

TABLE-2
FREE RADICAL SCAVENGING CAPACITIES OF THE METHANOLIC EXTRACT OF *D. biflorus* IN *in vivo* METHOD

Group	Superoxide dismutase (SOD) [unit /min/mg protein]			Glutathione peroxidase (GP_x) (mg of GSH consumed/min/mg protein)		
	Liver	Heart	Aorta	Liver	Heart	Aorta
Group I	12.55 ± 0.53 a**	15.58 ± 0.54 b*	22.09 ± 0.60 b**	15.41 ± 0.31 b*	17.78 ± 0.36 b*	23.45 ± 0.39 b*
Group II	7.91 ± 0.50 a**	6.94 ± 0.38 a*	11.69 ± 0.58 a*	8.55 ± 0.32 a*	8.27 ± 0.28 a*	11.13 ± 0.26 a*
Group III	10.26 ± 0.43 a**, b**	9.75 ± 0.57 a*, b*	19.50 ± 0.64 a*,b*	11.46 ± 0.31 a*, b*	11.03 ± 0.15 a*, b*	15.24 ± 0.57 a*, b*
Group IV	11.74 ± 0.35 a^{NS}, b*	11.65 ± 0.91 a^{NS}, b*	22.48± 0.29 ans,b*	12.25 ± 0.16a*, b*	12.67 ± 0.33 a*, b*	17.32 ± 0.13 a*,b*
Group V	12.55 ± 0.17 a^{NS}, b*	14.43 ± 0.28 a^{NS}, b*	23.67 ± 0.16 ± a**,b*	13.62 ± 0.35 a*, b*	14.75 ± 0.15 a*, b*	19.75 ± 0.20 a*,b*

Values are mean ± SE of six rabbits
p values : *< 0.001, **< 0.05 NS : Non significant
Group I : Control Group II : High Fat Diet (HFD)
Group III : HFD + methanolic extract of *D. biflorus* (200 mg/kg body wt/day)
Group IV : HFD + methanolic extract of *D. biflorus* (400 mg/kg body wt/day)
Group V : HFD + atorvastatin (1.2 mg/kg body wt/day)
a → group I compared with groups II, III, IV & V. b → group II compared with groups III, IV & V.

TABLE-3
FREE RADICAL SCAVENGING CAPACITIES OF THE METHANOLIC EXTRACT OF *D. biflorus* (LINN.) IN *in vivo* METHODS

Group	Glutathione-S-transferase (GST) (μ mole of CDNB-GSH-Conjugate to /min/mg/protein)		
	Liver	Heart	Aorta
Group I	24.91 ± 0.31b*	18.65 ± 0.28 b*	16.45 ± 0.18b*
Group II	9.05 ± 0.21 a*	8.24 ± 0.25 a*	7.40 ± 0.11 a*
Group III	15.54 ± 0.31 a*, b*	10.73 ± 0.24 a*, b*	9.47 ± 0.13 a*,b*
Group IV	17.90 ± 0.27 a*, b*	12.30 ± 0.29 a*, b*	10.45 ± 0.09 a*, b*
Group V	20.30 ± 0.25 a*, b*	17.35 ± 0.32 a*, b*	12.55 ± 0.16 a*, b*

Values are mean ± SE of six rabbits
Details of groups I – V are same as in Table-2.
a → group I compared with groups II, III, IV & V.
b → group II compared with groups III, IV & V.

Glutathione peroxidase (GP_x) detoxifies H_2O_2[19] and is involved in the reduction of a variety of hydroperoxides such as phospholipid hydroperoxides. Glutathione-S-transferase (GST) also involved in the metabolism of xenobiotics. Moreover literature studies reveal that atherogenic diet decreased the ratio of total/oxidized glutathione in tissue. The induction of glutathione peroxidase, glutathione-S-transferase by higher level of GSH may help to control hydroxy radical directly or indirectly.

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