ANTIOXIDANT ACTIVITY OF SOME INDIAN MEDICINAL PLANTS USED FOR THE TREATMENT OF DIABETES MELLITUS

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Summary

Oxidative stress may precede and accelerate the development of diabetes and then diabetic complications. Antioxidant action may be an important property of plant medicines associated with diabetes. So we analyzed antioxidant activity of the aqueous extracts of leaves of six medicinal plants namely *Gymnema sylvestre* (Asclepiadaceae), *Coccinia indica* (Cucurbitaceae), *Catharanthus roseus* (white), *C. roseus* (purple) (Apocynaceae), *Momordica charantia* var. *charantia* and *M. charantia* var. *muricata* (Cucurbitaceae) which are used to treat diabetes mellitus in India. The antioxidant activity was assessed in different systems of assay *e.g.* DPPH radical scavenging assay, superoxide scavenging assay, hydroxyl radical scavenging assay and assay to determine prevention of lipid peroxidation by plant extracts. The extracts were found to have different levels of antioxidant activity in the systems tested. Highest DPPH radical scavenging activity was found in *C. roseus* (white variety) IC_{50} value being 241.13 μg/ml. *C. roseus* (purple variety) (IC_{50} value 104.04 μg/ml) had highest superoxide radical scavenging activity. Highest hydroxyl radical scavenging activity was found in *C. indica* (IC_{50} value 552.93 μg/ml). IC_{50} value of *G. sylvestre* which prevented lipid peroxidation the most is 1390.07 μg/ml. Total antioxidant capacity equivalent to ascorbic acid and gallic acid was also determined. Total phenol content equivalent to gallic acid and total flavonoid content equivalent to catechin were determined. There was no correlation between total phenolics or flavonoids contents and antioxidant activity of the plants analysed. Although the antidiabetic property of *G. sylvestre* is due to gymnemic acid, present study reveals that the antioxidant property is due to the presence of phenolic compounds.

Key Words : Antioxidant activity; Diabetes; *Gymnema sylvestre*, *Coccinia indica*, *Catharanthus roseus*, *Momordica charantia* var. *charantia* and *M. charantia* var. *muricata*

Introduction

Oxidative stress is produced under diabetic conditions (1, 2) because hyperglycaemia depletes natural antioxidants and facilitates the production of free radicals (3). This chronic disease is associated with serious complications and a number of studies have suggested that enhanced oxidation is the underlying abnormality responsible for some of the complications in diabetes (4). Oxidative stress may also precede and accelerate the development of type 2 diabetes and then diabetic complications (2).

The World Health Organization (5) has estimated that by the year 2010 diabetes will affect 221 million people worldwide. Little work has been published regarding the dietary use of antioxidants. The leaves of *Gymnema sylvestre* R. Br. (Asclepiadaceae), *Momordica charantia* L. (Cucurbitaceae), *Coccinia indica* W. & A. (Syn. *Cephalandra indica* Naud.) (Cucurbitaceae) and *Catharanthus roseus* G. Don (*Vinca rosea* L.) (Apocynaceae) are used in India for the treatment of diabetes (6). The ability of these plants to lower the blood glucose level in experimental animals offer a degree of scientific validity to the traditional practices. Extract of *G. sylvestre* decreased the serum glucose concentration in dexamethasone induced hyperglycaemic mice (7). Ethanolic extract of *C. indica* leaves exhibited hypoglycaemic and hypolipidaemic effects in streptozotocin induced diabetic rats (8). The leaf juice of *C. roseus* produced dose dependent reduction in blood glucose of both normal and alloxan-induced diabetic rabbits (9) It has been suggested that antioxidant action may be an important property of plant medicines associated with diabetes (10). So we analyzed the free radical scavenging activity and the capacity of these plant materials to prevent lipid peroxidation *in vitro*.

Materials and methods

Reagents

Chemicals such as ethylenediamine tetra acetic acid (EDTA), trichloroacetic acid (TCA), butanol, ammonium molybdate, sodium dodecyl sulphate were puschased from E. Merck (India) Limited. 1,1 diphenyl-2-picrylhydrazyl and catechin were procured from Sigma, USA. Thiobarbituric acid (TBA) was purchased from Spectrochem PVT. Ltd., India. Nitroblue tetrazolium was obtained from Sisco Research Laboratories PVT. Ltd., India. All other reagents were of analytical grade.

Plant material

Leaves of *G. sylvestre* were collected from Puri. Leaves of *C. indica*, *C. roseus* (purple and white varieties), *M. charantia* var. *charantia*, *M. charantia* var. *muricata* were collected from Kolkata. Voucher specimens are deposited in the Department of Botany, University of Calcutta .

Extract preparation

The extracts prepared from the dried leaves were made by boiling in distilled water for 5 minutes and were used for analyzing antioxidant activity *in vitro*. The filtrates were used for the experiments. The dilution of the aqueous extracts were made in distilled water and were expressed in terms of weight/volume (µg/ml). The range of concentrations varied from 30 µg/ml to 6452 µg/ml depending on the plant material and their activity. Each experiment was repeated five times.

Each experiment was repeated five times.

DPPH radical scavenging activity

The method described by Braca et al., (11) was followed for determining the antioxidant activity of the extracts on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Aqueous extract was added 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated.

Assay of superoxide radical ($O_2^{-\cdot}$) scavenging activity

The method used by Martinez et al. (12) for determination of superoxide dismutase was followed after modification (13) in the riboflavin-light-nitrobluetetrazolium (NBT) system (14). The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

Assay of hydroxyl radical ($^{\cdot}OH$) scavenging activity

The assay was based on benzoic acid hydroxylation method as described by Chung et al. (15). Hydroxyl radicals are generated by direct addition of iron(II) salts to a reaction mixture containing phosphate buffer (16). Benzoate is hydroxylated to hydroxybenzoates. Benzoate is weakly fluorescent, but after monohydroxylation forms highly fluorescent products (17). The fluorescence was measured at 407 nm emission with excitation at 305 nm. Measurement of spectrofluorometric changes has been used to detect damage by hydroxyl radical.

Lipid peroxidation assay

A modified (13) thiobarbituric acid reactive species (TBARS) assay (18) was used to measure the lipid peroxide formed using egg yolk homogenates as lipid rich media (19). Lipid peroxidation was induced by $FeSO_4$. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm (20) which was measured. Inhibition of lipid peroxidation (%) by the extract was calculated.

Determination of total antioxidant capacity

Extract was combined with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95^{o}C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against a blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and gallic acid. The assay is based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH (21).

Determination of total phenol content

Phenol was determined by Folin-Ciocalteau reagent in alkaline medium and was expressed as gallic acid equivalent (22). Phenol content was calculated from the regression equations prepared from a range of concentrations of extract versus optical density for such concentrations and the regression equation prepared from different concentrations of gallic acid and optical density for the concentrations.

Determination of total flavonoid content

Total flavonoid content was determined following Kim *et al*. (23). Total flavonoids were expressed on a dry weight basis as μg/mg catachin equivalents.

Thin Layer Chromatography

Thin Layer Chromatography was performed on analytical plates (10x20 cm) precoated with silica gel 60 F_{254} (0.25 mm thick). Samples and standard compounds were applied on plates by linimat applicator (Camag, Multenze, Switzerland) and the plates were run in the following solvent systems

I Ethyl acetate : methanol : Formic acid :: 200 : 27 : 5 : 20

II Chloroform : ethyl acetate : formic acid :: 5 : 4 : 1

The plates were sprayed with DPPH solution, 5% $FeCl_3$ solution and Anisaldehyde-sulphuric acid (AS) reagent.

Statistical analysis

For determining the activity in the two varieties of C. roseus and M. charantia, experimental data were analysed using analysis of variance (ANOVA). There are two main effects namely, concentration and variety having 5 and 2 levels respectively. Also the interaction effect between these two factors is taken into consideration. The concentration factor is assumed to be random while the variety factor is treated as fixed.

Results

Percentage inhibition of free radical formation and percentage inhibition of lipid peroxide formation by aqueous plant extracts in different systems of assay were measured to assess the antioxidant activity of six Indian medicinal plants used for the treatment of diabetes mellitus. Total antioxidant activity equivalent to ascorbic acid and gallic acid were also determined. Considering 0% inhibition in the assay mixture without plant extract regression equations were prepared from the concentrations of extract and percentage inhibition. IC_{50} values (concentration of sample required to scavenge 50% free radical or to prevent lipid peroxidation by 50%) were calculated from the regression equation. IC_{50} values of these medicinal plants were compared in each system of assay (Fig. 1).

The free radical character of DPPH is neutralized by antioxidant (24). Aqueous extracts of all the plants neutralized free radical character of DPPH in a dose dependent manner (Table 1). *C. roseus* (white variety) shows the highest activity and the lowest IC_{50} value (241.13 μg/ml). *M. charantia* var. *charantia* failed to neutralize free radical character beyond 27% in the concentration range 1290 μg/ml to 6452 μg/ml.

Extracts of the Indian medicinal plants screened during the study inhibited formation of blue formazan due to reduction of NBT by $O_2^{-\cdot}$ and percentage inhibition is proportional to the concentration (Table 2). The activity of the plants determined by the IC_{50} values are in the order *C. roseus var*. purple (104.04 μg/ml) > *C. roseus* var. white (105.79 μg/ml) > *G. sylvestre* (143.02 μg/ml) > *C. indica* (418.16 μg/ml) > *M. charantia* var. *charantia* (478.29 μg/ml) > *M. charantia* var. *muricata* (478.34 μg/ml). All the plant extracts showed linear correlation between concentration of extract and hydroxyl radical scavenging activity (Table 3). Highest hydroxyl radical scavenging activity was found in *C. indica*. In other plants IC_{50} values are 713.45 μg/ml in *M. charantia* var. *charantia*, 884.46 μg/ml in *M. charantia* var. *muricata*, 1185.53 μg/ml in *G. sylvestre*, 6538.43 μg/ml in *C. roseus* (white variety) and 6826.38 μg/ml in *C. roseus* (purple variety). The two varieties of *C. roseus* have very low activity.

The aqueous extracts of the plants prevented lipid peroxidation in a dose dependent manner (table 4). IC_{50} value of *G. sylvestre* is 1390.07 μg/ml. In other plants IC_{50} values are 1592.18 μg/ml in *C. roseus* (white variety), 2024.77 μg/ml in *C. roseus* (purple variety), 1615.18 μg/ml in *M. charantia* var. *charantia*, 1800.85 μg/ml in *M. charantia* var. *muricata*, 1637.04 μg/ml in *C. indica*. Total antioxidant capacity of the tested plants are expressed as the number of equivalents of ascorbic acid and gallic acid (Table 5). *M. charantia* var. *charantia* has the highest total antioxidant capacity.

Some authors have reported a positive correlation between antioxidant activity and phenol content (13, 23). We have measured total phenol content and total flavonoid contentin the plants analysed but no relationship between the activity and content could be drawn. As *G. sylvestre* had high activity, the extract was further analysed. The aqueous extyract was further extracted three times each with ethyle acetate and n-butanol respectively to get aqueous fraction (AF), etrhyl acetate fraction (EAF) and n-butanol fraction (BF). With these fractions different radical scavenging activities were measured (Table 7). EAF was found to have highest DPPH radical scavenging activity followed by BF. But AF had the highest superoxide radical scavenging activity followed by BF. It has been reported previously that the ethyl acetate soluble fraction of *G. sylvestre* contained glycosides of kaempferol and quercetin and tamerixetin (25). AF, EAF and BF were chromatographed on three plates coated with thin layer silica gel. The three plates were sprayed with 5% $FeCl_3$ solution, AS reagent and 0.05% DPPH solution respectively, The pentacyclic triterpenoid gymnemic acid, usually isolated in the n-butanol fraction (26), is responsible for antidiabetic property of *G. sylvestre* (27). But the triterpenoids, giving coloration with As reagent did not scavenge DPPH radical. However the phenolic constituents giving blue-black colouration with 5% $FeCl_3$ solution scavenged DPPH radical in all the fractions. From the TLC studies it was found that the BF fraction contains phenolic compounds in addition to the triterpenoids. Although the antidiabetic property of *G. sylvestre* is due to gymnemic acid (27), present study reveals that the antioxidant property is due to the presence of phenolic compounds. Rutin, quercetin and kaempferol could be identified in *G. sylvestre* leaf extract after hydrolysis.

From ANOVA table it has been found that the null hypothesis of equality with respect to the effects (DPPH radical scavenging activity, $O_2^{-\cdot}$ Scavenging activity, OH radical scavenging activity and activity to prevent lipid peroxidation) of the two varieties of *C. roseus* is rejected with high p-value (0.99). The null hypothesis of equality with respect to the effects (OH radical scavenging activity and Prevention of lipid peroxidation) of two varieties of *M. chjarantia* is rejected with high p-value (0.99). But the null hypothesis of equality with respect to $O_2^{-\cdot}$ Scavenging activity of two varieties of *M. charantia* is accepted with p-value 0.301.

Table 1 : DPPH Radical Scavenging Activity

Plant material	Conc (μg/ml)	%inhibition± SD	Regression equation (r)[a]
C. indica	193.54 387.09 580.64 774.19 967.74	15.41 ± 2.52 34.32 ± 1.95 43.73 ± 0.65 50.82 ± 2.52 58.74 ± 1.74	y = 0.0604x + 4.598 (r = 0.9802)
C. roseus (purple variety)	96.77 193.54 290.32 387.09 483.87	18.47 ± 2.02 36.39 ± 2.93 48.00 ± 0.25 61.43 ± 3.72 77.00 ± 2.95	y = 0.1551x + 2.6806 (r = 0.9970)
C. roseus (white variety)	96.77 193.54 290.32 387.09 483.87	30.33 ± 3.46 31.33 ± 5.15 68.92 ± 3.04 82.33 ± 5.78 88.96 ± 2.43	y = 0.1888x + 4.4726 (r = 0.9719)
G. sylvestre	129 258 387 516 645	16.05 ± 3.02 29.85 ± 2.68 42.62 ± 2.80 54.87 ± 6.11 70.53 ± 3.88	y = 0.1067x + 1.2325 (r = 0.9990)
M. charantia Var *charantia*	1290.32 2580.64 3870.96 5161.29 6451.61	10.73 ± 0.20 17.60 ± 0.23 22.58 ± 0.29 24.73 ± 0.25 26.48 ± 0.22	
M. charantia Var *muricata*	129.03 258.06 387.09 516.12 645.16	14.02 ± 1.41 25.95 ± 1.27 37.05 ± 2.37 47.68 ± 1.64 64.05 ± 2.12	y = 0.0957x + 0.5779 (r = 0.9980)

[a] Correlation coefficient of dose response

Table 2 : Superoxide radical scavenging activity

Plant material	Con(μg/ml)	%inhibition± SD	Regression equation (r)[a]
C. indica	100	7.74 ±1.15	
	200	18.06 ± 0.46	y =0.1363x – 6.9953
	300	22.72 ± 1.35	
	400	37.01 ± 2.21	(r= 0.9262)
	500	76.90 ± 1.31	
C. roseus	33.33	13.60 ± 1.50	
(purple variety)	66.66	19.66 ± 2.23	y =0.4918x – 1.1683
	100.00	65.12 ± 2.63	
	133.33	66.70 ± 2.58	(r= 0.9513)
	166.66	73.81 ± 2.56	
C. roseus	33.33	3.85 ± 0.04	
(white variety)	66.66	15.55 ± 2.49	y =0.596x -13.05
	100.00	30.78 ±0.81	
	133.33	77.95 ± 2.66	(r=0.9521)
	166.66	91.55 ± 1.03	
G. sylvestre	66.60	29.16 ± 3.68	
	133.30	61.54 ± 4.49	y =0.2662x +11.9262
	200.0	73.92 ± 3.55	
	266.60	84.60 ± 1.03	(r= 0.9519)
	333.30	88.48 ± 0.78	
M. charantia	133.33	19.05 ± 1.13	
Var charantia	266.66	24.43 ± 1.98	y =0.1023x +1.0706
	400.00	43.57 ± 1.54	
	533.33	52.18 ± 1.28	(r = 0.9915)
	666.66	71.75 ± 1.29	
M. charantia	133.33	20.76 ± 0.87	
Var *muricata*	266.66	28.17 ± 1.22	y = 0.0982x +3.0261
	400.00	41.89 ± 3.88	
	533.33	55.97 ± 4.62	(r = 0.9946)
	666.66	67.80 ± 3.18	

[a] Correlation coefficient of dose response

Table 3 : Hydroxyl radical scavenging activity

Plant material	Con(μg/ml)	%inhibition ±SD	Regression equation (r)[a]
C. indica	200 400 600 800 1000	13.24 ± 1.38 41.36 ± 0.69 67.00 ± 8.86 74.66 ± 2.21 76.68 ± 3.40	y = 0.0848x +3.1114 (r =0.9639)
C. roseus (purple variety)	2000 4000 6000 8000 10000	7.95 ± 0.42 14.85 ± 0.49 45.43 ± 0.75 66.81 ± 0.75 75.43 ± 1.07	y = 0.0083x – 6.659 (r = 0.9762)
C. roseus (white variety)	2000 4000 6000 8000 10000	6.68 ± 0.08 16.64 ± 0.19 45.92 ± 0.74 64.82 ± 0.99 83.92 ± 1.41	y =0.0089x – 8.1921 (r = 0.9820)
G. sylvestre	500 1000 1500 2000 2500	23.58 ± 6.98 51.05 ± 3.73 68.80 ± 3.00 80.53 ± 4.55 89.91 ± 4.87	y =0.0365x + 6.7278 (r = 0.9809)
M. charantia Var. *charantia*	250 500 750 1000 1250	18.94 ± 2.53 35.30 ± 3.10 56.62 ± 0.90 67.78 ± 4.90 85.20 ± 2.77	y =0.0679x + 1.5562 (r= 0.9976)
M. charantia Var. *muricata*	250 500 750 1000 1250	24.86 ± 2.58 44.94 ± 7.60 44.82 ± 3.85 47.66 ± 8.89 66.56 ± 5.13	y = 0.0458 + 9.4915 (r = 0.9365)

[a] Correlation coefficient of dose response

Table 4 : Prevention of lipid peroxidation

Plant material	Con(μg/ml)	%inhibition± SD	Regression equation (r)[a]
C. indica	320.98 641.97 962.96 1283.95 1604.93	3.30 ± 0.14 17.46 ± 0.35 30.58 ± 0.50 41.03 ± 1.17 46.84 ± 0.67	y = 0.0321x – 2.5497 (r = 0.9889)
C. roseus (purple variety)	493.82 987.65 1481.48 1975.30 2469.13	17.00 ± 1.53 35.92 ± 1.11 41.17 ± 0.49 47.41 ± 0.56 55.81 ± 0.24	y = 0.0217x + 6.0621 (r = 0.9684)
C. roseus (white variety)	493.82 987.65 1481.48 1975.30 2469.13	31.06 ± 0.84 38.33 ± 1.02 50.84 ± 1.03 63.54 ±0.19 63.55 ± 0.21	y = 0.0247x + 10.673 (r = 0.9433)
G. sylvestre	312.50 625.00 1250.00 1875.00 2500.00	29.27 ± 2.85 35.97 ± 3.61 51.00 ± 2.50 60.03 ± 1.76 76.67 ± 1.97	y = 0.0265x + 13.183 (r = 0.9554)
M. charantia Var. *charantia*	444.44 888.88 1333.33 1777.77 2222.22	14.41 ± 1.51 31.23 ± 1.13 36.06 ± 1.03 46.41 ± 0.82 77.21 ± 3.94	y = 0.0313x – 0.5552 (r = 0.9684)
M. charantia Var. *muricata*	444.44 888.88 1333.33 1777.77 2222.22	16.02 ± 4.22 25.59 ± 3.62 33.95 ±2.28 50.13 ±1.08 62.19 ± 1.48	y = 0.0271x + 1.1967 (r = 0.9957)

[a] Correlation coefficient of dose respons

Table 5 : Total antioxidant activity

Plant material	Equivalent to ascorbic acid (μg)/mg plant material	Equivalent to gallic acid (μg)/mg plant material
C. indica	48.69	14.10
C. roseus (purple variety)	32.70	7.04
C. roseus (white variety)	25.96	8.29
G. sylvestre	84.02	23.13
M. charantia Var. *charantia*	2140.47	548.77
M. charantia Var. *muricata*	78.34	21.68

Table 6 : Phenol and flavonoid Content

Plant material	PHENOL CONTENT equivalent to gallic acid (μg) / mg plant material	FLAVONOID CONTENT equivalent to catechin (μg) / mg plant material
C. indica	961.9	7.929
C. roseus (purple variety)	1345.81	8.862
C. roseus (white variety)	1029.13	22.19
G. sylvestre	116.3	4.485
M. charantia Var. *charantia*	781.94	4.676
M. charantia Var. *muricata*	1034.36	13.282

Table 7: Antioxidant activity of different fractions of *G. sylvestre*

Fraction	DPPH rdical scavenging activity IC50 value (µg/ml)	Superoxide radical scavenging activity IC50 value (µg/ml)
AF	147.17	82.9
EAF	60.7	120.29
BF	268.63	183.29

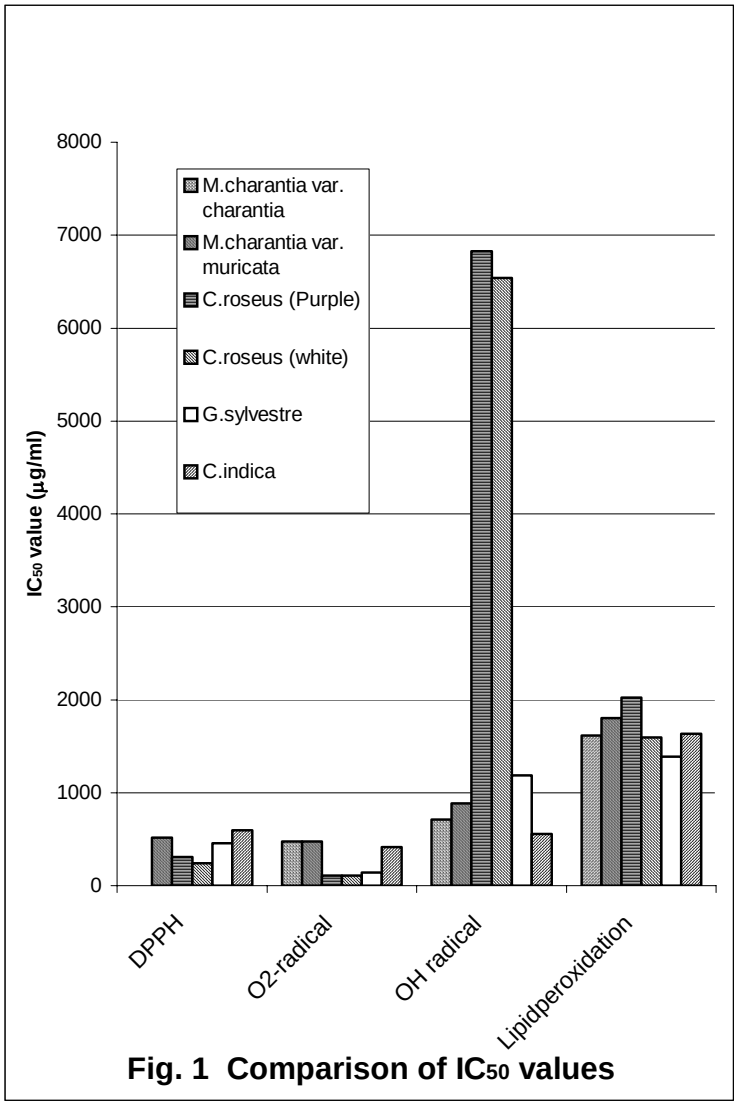

Fig. 1 Comparison of IC_{50} values

Discussion

The two varieties of *M. charantia* differ in fruit size (28). *M. charantia* var. *charantia* has large fruits, not tapering at both ends and *M. charantia* var. *muricata* has small fruits, tapering at both ends. Both fruits and leaves of *M. charantia* are used as a folk remedy for diabetes in India (6). Hypoglycaemic action of the fruits is repored in experimental animals (29 - 32). Feeding of *M. charantia* fruit extract reversed the streptozotocin induced glutathione-dependent oxidative stress related lipid peroxidation and glutathione-S-transferase activities (33). To our knowledge, no such report is known for *M. charantia* leaves. We report, for the first time, antioxidative activity of *M. charantia* leaves. It is also necessary to exmine the hypoglycaemic activity of the leaves in experimental animal.

The leaf juice of *C. roseus* produced dose-dependent reduction in blood glucose of both normal and diabetic rabbits (9). Oxygen radical absorbance capacity of this plant has been mentioned by Zeng and wang (34). We report here free radical scavenging activity of the two varieties of this plant – one variety producing white flowers and the other producing purple flowers. It is also necessary to examine if the plant protects a diabetic organism by inducing enzymatic antioxidants.

C. indica exhibits hypoglycaemic effect in streptozotocin induced diabetic rats (8). Oral administration of the leaf extract of this plant also reduced thiobarbituric acid reactive substances and hydroperoxides and significantly increased reduced glutathione, superoxide dismutase, catalase glutathione peroxidase and glutathione-S-transferase of streptozotocin diabetic rats (35, 36). This clearly shows the antioxidant property of *C. indica*. We report here free radical scavenging activity of this plant.

G. sylvestre leaf extract decreased the serum glucose concentration in dexamethasone induced hyperglycaemic animals (7). There is no report regarding the induction of enzymatic antioxidants after administration of this plant drug. We report here free radical scavenging activity of this plant *in vitro*.

Conclusion

The Indian medicinal plants examined during this study (except *M. charantia* leaf) have shown hypoglycaemic activity in experimental animals. Some of these plants increased enzymatic antioxidant level in experimental animals. We report here free radical scavenging activity of these plants. From the previous and present report it appears that these plants used for the treatment of diabetes may be helpful in reducing diabetic complications because of their antioxidative property. Further research in these regard is necessary.

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