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In vitro Antioxidant and Antidiabetic Activities of Wild and Micropropagated Plants Aqueous Extracts of *Caralluma bicolor* – An Endangered Medicinal Plant

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Abstract

Purpose: *Caralluma bicolor* is a succulent perennial herb growing wild in dry hill regions. It belongs to the family Asclepiadaceae. This species is endemic to the eastern slopes of the Western Ghats of the Coimbatore District, Tamil Nadu. The present study was carried out to assessment of its *in vitro* antioxidant and antidiabetic activities. **Methods:** *In vitro* antioxidant and antidiabetic activities in wild *C. bicolor* (WCB) and micropropagated *C. bicolor* (MCB) aqueous extracts studied through 1, 1-diphenyl-2-picrylhydrazil (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$), reducing power assay, hydroxyl radical scavenging activity, nitric oxide scavenging activity, and inhibition of α-amylase and α-glucosidase enzyme assays. **Results:** The highest antioxidant activities were observed in *C. bicolor* against in all five assays, namely, DPPH (IC_{50} = WCB 54.65 µg/mL, MCB 52.76 µg/mL), $ABTS^+$ (IC_{50} = WCB 71.04 µg/mL, MCB 69.10 µg/mL), reducing power assay (IC_{50} = WCB 62.25 µg/mL, MCB 53.90 µg/mL), hydroxyl radical scavenging assay (IC_{50} = WCB 60.93 µg/mL, 61.15 µg/mL), and nitric oxide scavenging assay (IC_{50} = WCB 70.20 µg/mL, MCB 74.75 µg/mL). Furthermore, in antidiabetic activity, the highest inhibition was observed in α-amylase and α-glucosidase enzyme activities and the IC_{50} values of WCB and MCB were 58.93, 60.55, 64.70, and 70.08 µg/mL, respectively. **Conclusion:** This study suggests that the aqueous extract of *C. bicolor* has great antioxidant and antidiabetic activities. The results revealed that the plant has rich in bioactive compounds, which serve as a novel therapeutic applications for drug discovery.

Keywords: *Caralluma bicolor*, Antioxidant, Antidiabetic, Aqueous extract, *In vitro*

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Introduction

Free radicals are extremely reactive and dangerous, which may also lead to vitamin deficiencies, anemia and malnutrition affect cognitive ability and intellectual development specified problems. This is able of damaging approximately all types of biomolecules such as lipids, proteins, carbohydrates, and nucleic acids. Oxidative stress can arise when cell cannot effectively destroy the excess free radicals produced.[1,2] Plants are the ancient source for medicinal uses. There is a rising interest in the quantity and use of plant antioxidant to medicinal plants with antioxidant properties for scientific research and industrial purposes.[3] These healthy protective effects attributed mostly to their polyphenolic components, mainly flavonoids and phenolic acids, which possess antioxidant activity against the reactive oxygen species.[3,4] The wild plants are regarded worldwide as an important area of the phytotherapeutic research in nutraceutical value and the antioxidant activity.[5]

Diabetes mellitus is one of the most serious diseases mostly accompanied by characteristic long-term complications and affects a major part of the world global populations.[6] According to the World Health Organization, the global prevalence of diabetes is 8.5% in the adult population. The International Diabetes Federation reported that 382 million people worldwide have diabetes mellitus.[7] 1.5 million deaths recorded in 2012 diabetes were the direct cause[8] and caused 2.2 million indirect deaths in additional, by growing the risks of cardiovascular and other diseases. This is a chronic disorder of metabolism that considered by high levels of glucose in the blood due to non-secretion of insulin or insulin sensitivity. It can be considered into diabetes type 1 which is an effect of insulin deficiency and diabetes type 2 is due to insulin resistance and the major factor contributing to the improvement of diabetes is resistance to insulin, which can result in hyperglycemia and dyslipidemia.[9] Chronic hyperglycemia is associated with exact organic complications that disturbing mainly eyes, kidneys, heart, and blood vessels.[10] A healthy diet and usual physical activity are well known to be useful to subjects at high risk of diabetes. Moreover, to avoid diabetes mellitus induced by oxidative stress, a good nutrition which is rich in antioxidants might also be helpful.[11] Actually, the treatment of diabetes mellitus synthetic hypoglycemic agents is available in the market. However, on chronic treatment, most of these conventional antidiabetic agents created some severe side effects. Therefore, the search and the identification of new safe and effective natural antidiabetic agents to treat diabetes is one of the most important ways to explore and continue to draw the attention of many researchers.[8] In fact, recent studies have been reported the hypoglycemic effect of many medicinal plants in experimental diabetes.[9,11-13]

The Asclepiadaceae family has genus *Caralluma* which are comprises about 200 genera and 2500 species. In India, *Caralluma*

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species are edible and form the part of traditional medicine system of the country.[14] The species of this family possess significant anti-inflammatory, antitumor, anticancer, antiulcer activity and cytoprotective, analgesic, antioxidant, hypolipidemic, antihyperglycemic, antidiabetic, treating paralysis and joint pain, and antipyretic properties.[15] The major constituents of *Caralluma* species is pregnane glycosides, saponin, and flavonoids.[16] *Caralluma bicolor* is an endemic succulent medicinal plant of South Western Ghats of Tamil Nadu. *C. bicolor* whole plant is used as a vegetable by the local folks. Hence, this study aims to evaluate the antioxidant properties and antidiabetic activity through α-amylase and α-glucosidase inhibition assays. With the increase in the challenge of staying healthy, need for new medicines will never end.

Materials and Methods

Plant Material Collection

C. bicolor plant was collected from Palamalai Hills, Periyanaickenpalayam, Coimbatore, Tamil Nadu, India. The collected plant of aerial parts dried in shade condition and powdered.

Plant Extracts Preparation

A 100 g powder of *C. bicolor* aerial part of wild *C. bicolor* (WCB) and micropropagated *C. bicolor* (MCB) plants was subjected to cold maceration method and the plant extracts collected and evaporated in room temperature and finally stored in amber bottle (light sensitive bottle) for further study.

In vitro Antioxidant Activity

1, 1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging activity

DPPH radical is scavenged by antioxidants by the donation of a proton forming the reduced DPPH.[17] Various concentrations of aqueous extract of the sample (4.0 mL) were mixed with 1.0 mL of methanolic solution containing DPPH radicals, ensuing in the last concentration of DPPH being 0.2 mM. The mixture was shaken forcefully and leaves to stand for 30 min, and the absorbance was calculated at 517 nm. The control was ascorbic acid. The proportion of inhibition in DPPH radical scavenging activity was intended as follows:

$$\% \text{ Inhibition} = A0\text{-}A1/A0 \times 100$$

2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radical scavenging activity

$ABTS^+$ decolorization assay requires the production of the $ABTS^+$ chromophore by the oxidation of $ABTS^+$ with potassium persulfate.[18] The reaction was initiated by the addition of 1.0 mL of diluted $ABTS^+$ to 10 μL of different concentrations (1–5 mg/mL) of extract and also to 10 μL of ethanol as a control. Positive control was ascorbic acid. The absorbance was examined at 734 nm after 6 min and the percentage of inhibitions was calculated. According to the equation, the inhibition was calculated,

$$I = A_o - A_1/A_o \times 100,$$

Where, A_o control reaction of absorbance and A_1 test compound of absorbance.

Reducing Power Assay

Reducing power was measured by direct electron donation in the reduction of Fe^{3+} (CN-)6 toFe^{2+}(CN-)6.[19] The reaction mixture contained 2.5 mL of different concentrations of the sample, 2.5 mL of 1% potassium ferricyanide, and 2.5 mL of 0.2 M sodium phosphate buffer. The control restricted all the reagents apart from the sample. The mixture was incubated at 50°C for 20 min and was determined by the addition of 2.5 mL of 10% (w/v) of trichloroacetic acid followed by centrifugation at 3000 rpm for 10 min. 5.0 mL of the supernatant upper layer was mixed with 5.0 mL of deionized water and 1.0 mL of 0.1% ferric chloride. Seven hundred nanometers were calculated the absorbance at against blanks that contained phosphate buffer and distilled water. The improved reducing power of the sample indicates increased absorbance.

Hydroxyl Radical Scavenging Assay

The hydroxyl radicals were produced by $FeSO_4$ and hydrogen peroxide and recognised by their capacity to hydroxylate salicylate.[20] A reaction mixture of 3.0 mL volume contained, 1.0 mL of 1.5 mM $FeSO_4$, 0.7 mL of 6 mM hydrogen peroxide, 0.3 mL of 20 mM sodium salicylate, and 1.0 mL of different concentrations (5–100 μg/ml) of sample. Following incubation for an hour at 37°C, the absorbance of the hydroxylated salicylate complex was calculated at 562 nm. The positive control used for Vitamin E. The effect of percentage scavenging was calculated as,

$$\text{Scavenging activity} = [1-(A_1-A_2)/A_0] \times 100$$

Where, A_o is absorbance of the control, A_1 is absorbance in the presence of the sample, and A2 is absorbance without sodium salicylate.

Nitric Oxide Scavenging Assay

The interaction of sample with nitric oxide was checked by the nitride detection method.[21] Nitric oxide generated as of sodium nitroprusside in aqueous solution by physiological pH interacts with oxygen to make nitrite ions which were calculated at 540 nm. The reaction mixture (6.0 mL) containing sodium nitroprusside (4.0 mL), phosphate buffer saline (1.0 mL), and different concentrations (5–100 μg/mL) of a leaf extract (1.0 mL) in DMSO was incubated at 25°C for 15 min after incubation, 0.5 mL of the reaction mixture containing nitrite was removed, 1.0 mL of sulfanilic acid reagent was added, mixed well, and allowed to stand for 5 min for completion of diazotization, and 1.0 mL of naphthyl ethylene diamine dihydrochloride was further, mixed well, and approved to stand for 30 min in gentle light. A pink-colored chromophore was formed. The absorbance of these solutions was consistent at 540 nm against subsequent blank solutions. Rutin was used as a standard.

The inhibition was calculated according to the equation,

$$I = A_o - A_1/A_o \times 100,$$

Anywhere, A_o is absorbance of control response and A_1 is absorbance of test compound.

In vitro Antidiabetic Activity

α-*amylase inhibition assay*

Various concentrations of the plant extracts and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) consist of porcine pancreatic α-amylase enzyme (EC 3.2.1.1) (0.5 mg/mL) were incubated at 25°C for 10 min.[22] Following the incubation, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was supplementary to the reaction combination. At last, the reaction mixture was incubated at 25°C for 10 min, result by adding of 1.0 mL of dinitrosalicylic acid. Surely, the reaction was stopped by incubation in boiling water for 5 min and cooled at room temperature. The reaction mixture was diluted with 10 mL distilled water, and the absorbance was measured at 540 nm in a spectrophotometer. The combination of all other reagents and the enzyme apart from the control the sample was used. The percentage of inhibition α-amylase inhibitory activity was asserted as,

$$\text{Inhibition } (\%) = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{Control}}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of α-amylase activity under assay condition.

α-*glucosidase inhibition assay*

Different volume of plant extracts (5–100 µg/mL) and 10 µL of α-glucosidase (0.5 mg/mL) in 0.1 M phosphate buffer (pH 6.9) solution were incubated at 25°C for 10 min.[23] Then, 50 µL of 5M p-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer (pH6.9) solution was added. Reaction mixtures were incubated at 25°C for 5 min and the absorbance was taken at 405 nm by a spectrophotometer. The mixture of all other reagents and the enzyme except the sample was used as a control and the results of α-glucosidase inhibition activity were expressed in terms of inhibition percentage. The percentage of α-glucosidase inhibitory activity is calculated by the following formula:

$$\text{Inhibition } (\%) = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{Control}}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of α-glucosidase activity under assay condition.

Statistical Analysis

All values obtained are an aggregate of three replicates, and it was denote as mean ± standard deviation. Every recorded data were analyzed with the help of the statistical tool of the Window's statistical software package SPSS (release 8.0). The test of importance was conducted by one-way analysis of variance.

Results And Discussion

In vitro Antioxidant Activity

DPPH radical scavenging activity

In DPPH assay and graphical representation of *C. bicolor,* WCB and MCB plant extracts were presented [Figure 1]. WCB plant extract had significant scavenging activity in all the concentrations. DPPH is a constant free radical that produces in ethanol a violet solution. The antioxidant compounds decrease these radicals and produced a neutral complex. Development of a colorless complex depends on the hydrogen donating potential of antioxidant compounds.[24] The MCB plant extract showed the highest inhibition percentage 65.75 at 1000 µg/mL concentration followed by 52.34 at 800 µg/mL and in WCB plant extract has the highest scavenging activity at 58.43 at 1000 µg/mL and 51.63 at 800 µg/ml concentrations. IC_{50} value of the extracts was 54.65 and 52.76, respectively, against the control ascorbic acid 54.32 [Figure 1]. The DPPH scavenging activity was almost equal with MCB plant extract. However, these results are comparable to previous results of *C. fimbriata* methanol ($IC_{50:}$ 50 µg/mL), ethanol ($IC_{50:}$ 200 µg/mL), ethyl acetate ($IC_{50:}$ 900 µg/mL), and aqueous extract ($IC_{50:}$ 235 µg/mL).[25] *Caralluma tuberculata* was revealed as a good quality basis of antioxidants next to diabetes induced oxidative stress in animal model, which can restore the activity of antioxidant enzymes and oxidative markers to near-normal levels.[26] Based on the results of the current study, both WCB and MCB plant extracts showed notable DPPH radical quenching effects at all tested concentrations. The study findings were in line with those of the previous studies reporting significant antioxidant activity of *Caralluma flava* and *Caralluma arabica* using the DPPH test.[27,28] Furthermore Rehman *et al.*,[24] reported that the callus of *C. tuberculata* has polar fractions and had more DPPH scavenging property compared to non-polar fractions.[29] Furthermore Saeed *et al.*,[30] reported that extracts with high flavonoid and phenol compounds showed potent DPPH scavenging activities.

IC_{50} value of WCB sample: 54.65 µg/mL
IC_{50} value of MCB sample: 52.76 µg/mL
IC_{50} value of ascorbic acid (standard): 54.32 µg/mL

$ABTS^+$ Radical Scavenging Assay

$ABTS^+$ radical scavenging activity of *C. bicolor* extracts is reported in Figure 2. From this Figure 2. It was observed that when the concentration increases the value of the absorbance also increased. The absorbance is 61.36 and 63.20 at the concentration 100 µg/mL in WCB and MCB extracts, whereas the standard Vitamin C absorbance is 61.45 of WCB and MCB extracts, respectively. The inhibition of both samples is equal. The second best inhibition was recorded in 75 µg/mL concentration with 51.63 and 54.20, respectively, against the control value is 53.30. $ABTS^+$ cation radical scavenging activity decolorization assay associated lipophilic and hydrophilic antioxidants together, and carotenoids flavonoids,

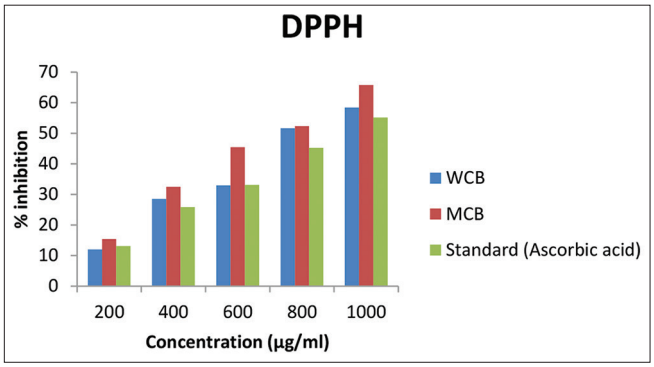

Figure 1: 1, 1-diphenyl-2-picrylhydrazil activity of wild and *in vitro* plant extract of *Caralluma bicolor*

hydroxycinnamates, as well as plasma antioxidants. The preformed radical monocation of $ABTS^+$ is generated in oxidation of $ABTS^+$ by potassium persulfate and is concentrated in the presence of such hydrogen donating antioxidants. The activity of the testing sample extract is expressed as a micromolar corresponding of butylated hydroxytoluene. The major plant compounds with antioxidant activity are polyphenols, while they are not the only ones. The antioxidant activity of the phenolic compounds is substantially due to their redox properties.[31,32] This can play a major role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. In the present study result IC_{50} value of WCB sample is 71.04 µg/ml, IC_{50} value of MCB sample is 69.10 µg/ml and IC_{50} value of vitamin C (standard): 57.44 µg/ml. Whereas methanol and aqueous extract of Asclepiadaceae member *Hemidesmus hamiltonii* on $ABTS^+$ cation radical scavenging activity IC_{50} value was 1108.9 and 2720.1 µmol/g.[33]

IC_{50} value of WCB sample: 71.04 µg/ml
IC_{50} value of MCB sample: 69.10 µg/ml
IC_{50} value of Vitamin C (standard): 57.44 µg/ml

Reducing Power Assay

The reducing power assay results recorded from WCB and MCB plant extracts and its graphical representations are presented in Figure 3. In the five concentrations of WCB and MCB extracts are examined (5 µg/mL–100 µg/mL), the highest percentage 67.36 and 73.30% was observed at 100 µg/mL and the second best concentration 51.63 and 61.40% was observed at 75 µg/mL against the control 57.50. Reducing power activity is frequently used to assess the capacity of natural antioxidant to donate electron.[34,35] Many reports have exposed that there is a direct association between antioxidant activities and reducing power of particular plant extracts.[36,37,34] The IC_{50} value of WCB and MCB plant samples 62.25 and 53.90 and the control Vitamin C is 57.54 [Figure 3]. This results agreeing with earlier findings of *Caralluma europaea*.[38] The reducing power activity increased consistently with the increase in the volume of extract concentration. It is known further that the reducing power activity of WCB and MCB plant extracts was more or less similar to the standard ascorbic acid.

IC_{50} value of WCB sample: 62.25 µg/mL
IC_{50} value of MCB sample: 53.90 µg/mL
IC_{50} value of Vitamin C (standard): 57.54 µg/mL

Hydroxyl Scavenging Assay

The hydroxyl radical scavenging of WCB and MCB plant extracts of *C. bicolor* and its graphical representation is shown in Figure 4. The $OH^{\cdot}$ radical has capacity to join nucleotides in DNA and source strand breakage, which contributes to mutagenesis, carcinogenesis, and cytotoxicity. In accumulation, this species is measured to be one of the rapid initiators of the lipid peroxidation process, abstracting hydrogen atoms beginning unsaturated fatty acids.[39] As per results, the higher percentage inhibition of WCB and MCB extracts was 75.50 and 65.50 at the concentration of 100 µg/mL. This is followed by 65.5 and 52.5 at 75 µg/mL concentration, respectively. The IC_{50} values in WCB, MCB plant extracts, and Vitamin E were 61.93, 60.15, and 57.54 µg/mL, respectively [Figure 4]. This study reports similar to *C. adscendens* var. *fimbriata* methanol extracts.[40]

IC_{50} value of WCB sample: 61.93 µg/mL
IC_{50} value of MCB sample: 60.15 µg/mL
IC_{50} value of Vitamin E (standard): 57.54 µg/mL

Nitric Oxide Scavenging Assay

The results observed for the nitric oxide scavenging assay and the graphical representation is obtained in Figure 5. In the present study, WCB and MCB plant extracts of *C. bicolor* at the concentration of 5, 25, 50, 75, and 100 µg/mL were studied in the nitric oxide scavenging activity. It is postulated that reactive nitrogen species such as NO or peroxynitrite (ONOO-) contribute to the development of a number of diseases.[41] All the concentrations of both extracts have excellent inhibition percentage. Among

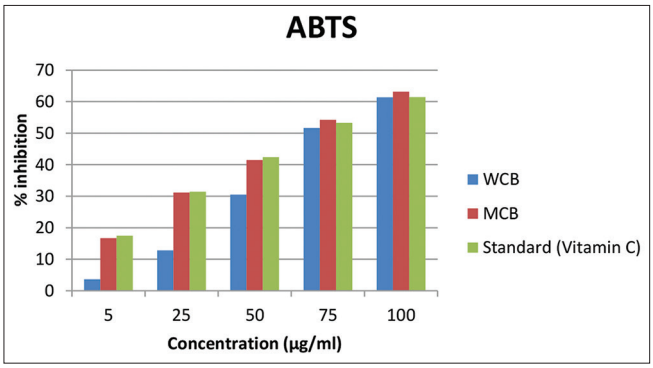

Figure 2: 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay of wild and *in vitro* plant extract of *Caralluma bicolor*

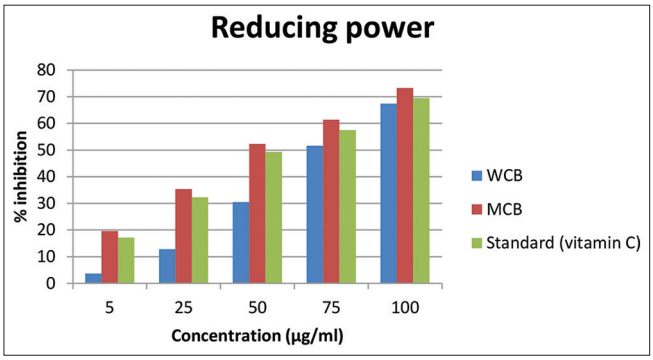

Figure 3: Reducing power assay of wild and *in vitro* plant extract of *Caralluma bicolor*

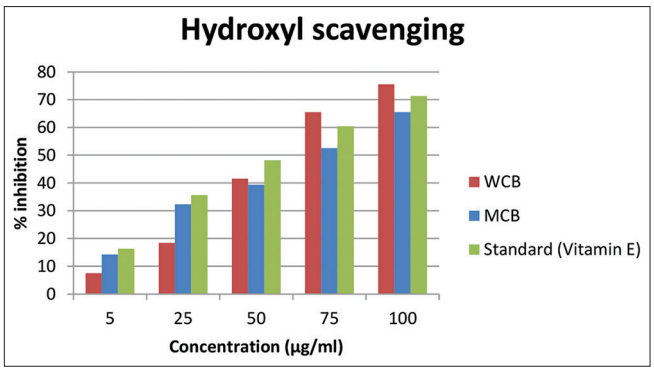

Figure 4: Hydroxyl scavenging assay of wild and *in vitro* plant extract of *Caralluma bicolor*

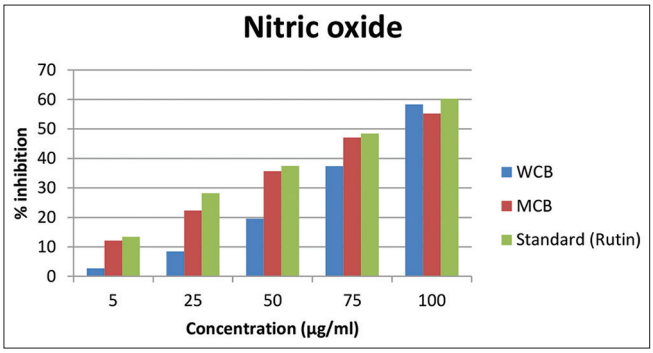

Figure 5: Nitric oxide scavenging assay of wild and *in vitro* plant extract of *Caralluma bicolor*

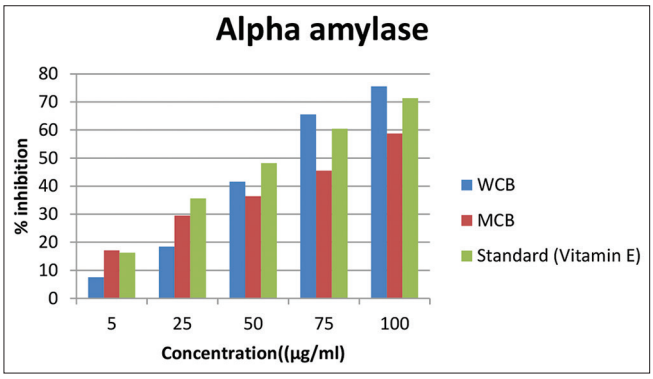

Figure 6: Inhibition of α-amylase enzyme activity of wild and *in vitro* plant extract of *Caralluma bicolor*

this concentration, the highest inhibition percentage 58.33 and 55.20 was recorded at 100 µg/mL concentrations, respectively. The second best inhibition was observed 47.10 and 37.4 at 75 µg/mL concentration [Figure 5]. The IC_{50} value calculated using linear regression analysis was found to be 70.20, 74.75, and 77.43 in WCB, MCB, and standard rutin, respectively. Almost similar results of radical scavenging activity were observed in *C. flava* extract.[28] In *C. bicolor* plant extracts, nitrogen reduction capacity was noticed as extracts concentration-dependent manner.

IC_{50} value of WCB sample: 70.20 µg/mL
IC_{50} value of MCB sample: 74.75 µg/mL
IC_{50} value of rutin (standard): 77.43 µg/mL

In vitro Antidiabetic Activity

Inhibition of α-amylase enzyme activity

The WCB and MCB plant extracts have significant inhibitory action of α-amylase enzyme. The values and graphical representation are shown in Figure 6. It shows that the extracts showed a concentration dose-dependent manner. A diabetic is group of metabolic disorders and leads to the most important causes of the death all over the world. Numerous curative agents are available to treat diabetics, but they are costly and also produced many side effects.[42] Conservative medicinal plants having antidiabetic activities can give valuable source for the finding of safer hypoglycemic agents.[43] The highest percentage 75.50 ± 0.17, 58.75 ±0.20, and 71.35 ± 0.85 were observed at concentration of

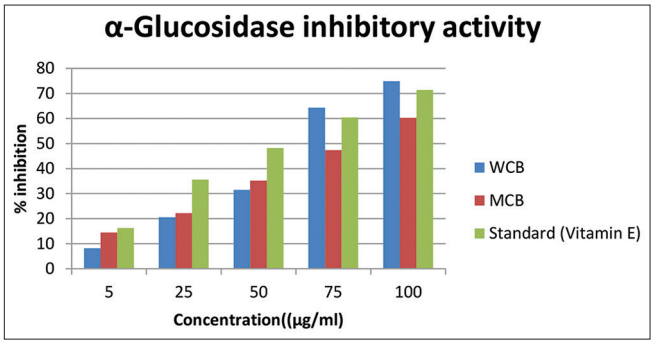

Figure 7: Inhibition of α-glucosidase enzyme activity of wild and *in vitro* plant extract of *Caralluma bicolor*

WCB, MCB, and Vitamin C extracts, respectively [Figure 6]. The IC_{50} value was observed to be 58.93, 60.55, and 57.54 µg/mL, respectively. Almost similar activities were observed the plant extract of *C. europaea*.[44]

IC_{50} value (µg/ml) WCB sample= 58.93 µg/mL
IC_{50} value (µg/ml) MCB sample= 60.55 µg/mL
IC_{50} value (µg/ml) Vitamin C (Standard)= 57.54 µg/mL

Inhibition of α-Glucosidase Enzyme Activity

In the present study, the α-glucosidase enzyme activity of WCB and MCB plant extracts of *C. bicolor* was carried out. The results of all the extracts are shown in Figure 7 which shows when the concentration increases, the inhibition percentage also increased in α-glucosidase enzyme activity. The highest percentage of inhibition 74.80 ± 0.17 and 60.20 ± 0.30 was observed at 100 µg/ml concentration of WCB and MCB extracts respectively. The extracts have the highest percentage of inhibition activity against the control Vitamin E at the concentration of 100 µg/ml which was observed 71.35 ± 0.85 [Figure 7]. But compare to the standard, the plant extracts showed higher activity. It shows that the extracts have concentration dose-dependent manner. This might be due to the nature of extract constituents (alkaloids, flavonoids, saponins, phenols, terpenoids, and steroids) present in the both solvent extracts and could be responsible as being efficient inhibitors of α-amylase and α-glucosidase.

IC_{50} value (µg/ml) WCB sample= 64.70 µg/mL
IC_{50} value (µg/ml) MCB sample= 70.08 µg/mL
IC_{50} value (µg/ml) Vitamin C (Standard)= 57.54 µg/mL

Conclusion

The study suggests that crude extract possesses promising antioxidant and antidiabetic activity; thus, organic antioxidant and antidiabetic medicines can be generated for possible therapeutic applications. There is no report on this plant species. This is the fundamental collective report on antioxidant and antidiabetic activity on the aerial parts of *C. bicolor*. The results demonstrated that the antioxidant and antidiabetic activities of aqueous extracts of both WCB and MCB plants have good results. Hereby, we propose the efficacy of *C. bicolor* as a safe antioxidant and antidiabetic agent. Our result opens up the possibility in the future to identify the potential therapeutic agents from *C. bicolor* for the development of herbal-based medicine.

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