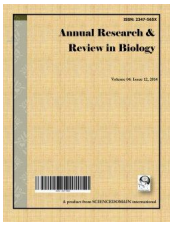

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In vitro Antiglycation, Antioxidant Properties of *Coleus forskohlii "Balady"* Leaves and Stem and their Antioxidant Enzyme Activities

Yaaser Q. Almulaiky[1,2,3], Abudukadeer Kuerban[4*], Faisal Aqlan[3,5], Saeed Ahmed Alzahrani[6], Mohammed N. Baeshen[1], Mohamed Afifi[3,7,8], Ammar Alfarga[1], Waleed Al-Shawafi[2,4] and Mohammed Alkhaled[1,8]

[1]Department of Biochemistry, Faculty of Science, University of Jeddah, Jeddah, Kingdom of Saudi Arabia.
[2]Chemistry Department, Faculty of Applied Science, Taiz University, Yemen.
[3]Center of University of Jeddah for Science and Medical Research, Kingdom of Saudi Arabia.
[4]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.
[5]Chemistry Department, Faculty of Science, University of Jeddah, Jeddah, Kingdom of Saudi Arabia.
[6]Medical College of Al-Baha University, Al Baha, Kingdom of Saudi Arabia.
[7]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Egypt.
[8]Department of Biological Science, Faculty of Science, University of Jeddah, Kingdom of Saudi Arabia.

Authors' contributions

This work was carried out in collaboration between all authors. Authors YQA and AK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors FA, SAA and MNB managed the analyses of the study. Authors MA and AA managed the literature searches. Author WA performed fluorescence analyses. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: abdukadir830@gmail.com;*

ABSTRACT

Aims: In this study, the antiglycation and antioxidant potency of methanol (80%) extracts, as well as the antioxidant enzyme activities from both leaves and stem of *Coleus forskohlii "balady"*, were analyzed.
Place and Duration of Study: Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, between February 2017 and July 2017.
Methodology: Antiglycation activity was evaluated by BSA-glucose glycation assay. The total phenolics, flavonoids, chlorophyll, carotenoid, lycopene content were estimated, and antioxidant activity was tested by the capacity to reduce ABTS reducing power assay, DPPH radical-scavenging activity assay, FRAP assay. Enzyme activities from both leaves and stem of *Coleus forskohlii* were tested for polyphenol oxidase, peroxidase, and catalase.
Results: Results indicated that methanolic extracts from leaves exhibited strong antiglycation and antioxidant activities than from stem. Among the enzymatic antioxidant properties, except for the activity of peroxidase, the activities of catalase and polyphenol oxidase were significantly higher ($P < 0.05$) in leaves than in stem.
Conclusion: It can be concluded that the leaves contained significantly rich sources of both enzymatic and non-enzymatic antioxidants can be used as a natural antioxidants.

Keywords: Methacrylamido phenylboronic acid; antioxidant; antiglycation; enzyme; coleus forskohlii "Balady"; stem; leaves.

1. INTRODUCTION

Coleus Forskohlii "Balady" is one of the family members of the Lamiaceae; a large family that is common, widely spread and mostly distributed in the Mediterranean region [1]. It is native to Al-Baha and Taif mountains in Saudi Arabia. Seven different species were described In the Flora of Saudi Arabia [2]. It has been prescribed in a folk medicine to treat various medical conditions including stomach, liver, intestine disorders, heart, and respiratory problems [3]. Also, it is cultivated as ornamental. Its leaves were used as antiseptic, and deodorant dressing for wounds and its leaf extract was used to treat ear infections [4]. The different species of this plant have been reported to contain rosmarinic acid and also flavonoid glucuronides and diterpenoids. Invitro antioxidant, antimicrobial and antiacetylcholinesterase activity as well as in vivo hypotensive activity were investigated [5,6]. Nowadays, *Coleus forskohlii* is used as a fat burning supplement. It increases cellular levels of a molecule called cyclic adenosine monophosphate (cAMP). Elevated cAMP levels are associated with increased rates of fat loss, and can improve the effects of other fat burning compounds [7]. It was reported that *Coleus forskohlii* supplementation implicated to increase the testosterone level, and thus protect against cancer and inflammation, these data demonstrate the therapeutic potency of this plant.

A feature of diabetes is that the rate of protein glycation and the formation of advanced glycation endproducts (AGEs) increases spontaneously due to the abnormally elevated level of sugar in the blood. The Maillard reaction, non-enzymatic glycation after a complex series of reactions that involve reducing sugars and proteins, produces a large number of end-products that are known as AGEs. AGEs are related to the pathogenesis of many diseases such as diabetes, diabetic complications (retinopathy, neuropathy, atherosclerosis, end-stage renal diseases, rheumatoid arthritis and neurodegenerative diseases [8]. Nowadays there is an increasing demand for natural AGEs inhibitors for curing diabetes that have fewer side effects compared to synthetic drugs [9].

The use of traditional medicine is wide spread around the world and plants are still a large source of natural antiglycants and antioxidants for development of novel drugs [10]. They might be used as preventive modern medicine. To data, two-thirds of the world's plant species have medicinal value; in particular, many medicinal plants have great antiglycation and antioxidant potential [11]. Despite several studies have been focused on the antioxidant activity of *C. forskohlii* from different countries [12]. The antiglycation and antioxidant activity have not yet been established for *C. forskohlii* Balady in Saudi Arabia. Therefore, the aim of this study was to evaluate in vitro antiglycation, antioxidant effect and antioxidant enzymes of *C. forskohlii* Balady.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Coleus. forskohlii "Balady" is a wild plant as it shown in Picture 1. Leaves and stem of this plant were collected from Al-Baha city, Saudi Arabia, May, 2017, N 20.231572, S 41.130992, and identified by an expert of the biology department, AL-Baha University.

Picture 1. *Coleus. forskohlii "Balady"*

2.1.2 Chemicals

Methacrylamido phenylboronic acid (MPBA), 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) were purchased from Fluka (Germany). Guaiacol, Hydrogen peroxide, and catechol were purchased from Sigma (USA). Other chemicals were of analytical grade.

2.2 Methods

2.2.1 Preparation of solvent extracts

Coleus. forskohlii "Balady" Leaves and stem were washed by tap water after that rinsed in distilled water. They were cut into pieces, dried overnight in hot air oven at 60°C, ground separately to powder. 5 grams of each ground plant materials were shaken individually in 80% methanol for 24 h on the shaker at 150 rpm, 25ºC. Extracts were filtered using Whatman filter paper No. 1. The filtrate designated as methanol extract, and removed methanol by Buchi Rotavapor R-300 Evaporation Systems, remaining water was removed by lyophilization, The extracts were weighed (0.74 g for stem and 0.81 g leaves) and stored at 4°C in storage vials for experimental use.

2.2.2 Antiglycation properties of the leaves and stem extract

The antiglycation effect of the leaves and stem on glycated BSA were determined as described by M. Kazeem [13] with slight modifications. AGEs formation was developed in a 10 mL phosphate buffered (50 mmol /mL, pH 7.4) reaction mixture in which the final concentration of BSA was 10 mg/mL. The concentration of glucose was 15 mmol/mL, and 0.02% sodium azide was added as an antibacterial agent. The reaction mixtures were kept in the incubator at 37℃ with gentle shaking for 15 days. Group C only with BSA was kept as a negative control for AGEs formation. Group B contained BSA with Glucose was kept as a positive control for AGE formation. Group A only with Glucose was kept as a control group for glucose reduction. Group S and L with 5mg/mL concentration of each were held for evaluation of the antiglycation effect of stem and leaves extract. AGE formation and glucose reduction were assayed at first day, seventh day and fifteenth day. For measuring AGEs product, 0.5 mL TCA (100%) was added to 0.5 mL reaction mixture and centrifuged (15,000 rpm) for 5 minutes at 4℃. After centrifugation, the pellet was washed with 0.5 mL 5% TCA. The supernatant containing glucose, methanol extract, and interfering substance, was removed and the pellet contained (AGEs)-BSA was dissolved 1.5 mL PBS (pH 10). AGEs formation was measured by the fluorescence intensity at a wavelength of 370 nm for excitation and 447 nm for emission by using the spectrofluorometer RF-1500 (Shimadzu, Japan). Glucose was assayed by 3, 5-Dinitrosalicylic acid method [14]. Briefly 0.5 mL of an alkaline solution of 3, 5-dinitrosalicylic acid was added to 0.5 mL of the reaction mixture and incubated at 97℃ for 30 minutes, and absorbance was read at 540 nm against blank.

2.2.2.1 Analysis of protein glycation by MPBA gel electrophoresis

0.5% MPBA polyacrylamide gels were prepared according to Marta P. Pereira Morais [15] briefly 0.5% of MPBA was added to the electrophoresis gel preparation solution before adding 10% SDS, ammonium persulfate and TEMED. Vortexed to dissolve. Polyacrylamide resolving gels were polymerized, the stacking gel, containing no MPBA, cast on top the resolving gel. The protein samples were applied to the stacking gel in sample buffer (2% w/v SDS, 2 mM DTT, 15% glycerol, 100 mM Tris pH 6.8 and bromophenol

blue) and gels were electrophoresed at 60 mA for 60 min in glycine buffer (25 mM Tris, pH 8.3, 250 mM glycine and 0.1% SDS) at room temperature.

2.2.3 Estimation of the total phenolic contents

Total phenolic content was measured according to Velioglu et al. [16]. 10 µL (1 mg/mL) of the methanol extract was mixed with 100 µL Folin-Ciocalteu reagent and 890 µL of distilled water and kept for 5 min at room temperature. 0.5mL of 20% sodium carbonate was added and allowed to react for 30 min. Absorbance was recorded at 750 nm. Total phenols were quantified from a standard curve obtained from Gallic acid. The results expressed as mg GAE /g tissues.

2.2.4 Estimation of the total flavonoid contents

The total flavonoids content of the extracts were determined using a modified colorimetric method described by Zhishen et al. [17] Catechin was used as a standard. The 0.25 mL Methanol extract was mixed with 1.25 mL distilled water and 75 µL, 5% $NaNO_2$ solution. After standing for 6 min, the mixture was combined with 0.15 mL, 10% $AlCl_3$ solution. 1 M NaOH (0.5 mL) and distilled water (275 µL) was added to the mixture 5 min later. The absorbance of the solutions was recorded at 510 nm. Total flavonoids were calculated from the catechin acid calibration curve (Fig. 6). The results expressed as mg CE/g tissues.

2.2.5 Antioxidant assays

2.2.5.1 DPPH radical scavenging activity

Free radical scavenging activity of crude methanol extracts was determined using the DPPH radical scavenging assay [18]. 0.1 mL methanol extracts were added to 0.9 mL freshly prepared DPPH (0.1 mM) reagent and kept in the dark at 25 ℃ for 30 min. An equal amount of methanol was used as a control. The absorbance was recorded at 517 nm using a spectrophotometer. Scavenging Activity (%) was calculated using the following formula:

DPPH radical scavenging %= [1–(OD sample/OD control)] × 100.

The results were plotted as the % of scavenging activity against the concentration of the sample. The IC_{50} value, required for providing 50% free radical scavenging activity was calculated from the plots.

2.2.5.2 ABTS radical cation decolorization assay

ABTS•+scavenging activity of methanol extracts was determined according to the method of N. Pellegrini [19]. The ABTS•+ solution was diluted to get an absorbance of 0.750 at 734 nm in PBS (pH 7.4). 1 mL of ABTS•+ solution was added to 0.5 mL crude methanol extract. The absorbance was recorded 1 min after mixing, and the percentage of radical scavenging was calculated relative to a blank containing no scavenger. The scavenging activity of test compounds was calculated using the following equation:

ABTS·+scavenging (%) = [1–(OD sample/OD control)] × 100.

IC_{50} was defined as the amount of methanol extract required for 50% of free radical scavenging activity.

2.2.5.3 Ferric Reducing antioxidant power assay (FRAP)

The ferric reducing power was determined according to the method of Oyaizu [20]. Various concentrations of 2.5mL methanolic extracts were mixed with 2.5 mL of 0.2 M PBS (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated at 50 ℃ for 20 min. After 2.5 mL of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 3000 rpm for 10 min. From the upper layer, 5mL was mixed with 5 mL deionized water and 1 mL of 0.1% of ferric chloride. The absorbance was measured at 700 nm. The extract concentration providing 0.5 AU of absorbance (EC50) was calculated from the graph of absorbance at 700 nm against extract concentration.

2.2.6 Determination of Chlorophyll, Carotenoids, and Lycopene

Total carotenoids were determined by a method described by Wang et al. [21]. One-tenth homogenate in 80% methanol (10 mL) was prepared as described above. This homogenate was successively extracted by addition of 5 mL hexane until the solvent became colorless. The volume was adjusted to 25 mL of, appropriately diluted and absorbance was measured at 450, 645, and 663 nm. The following formulae were used to calculated total carotenoids, lycopene, and chlorophylls:

Chlorophyll (µg/mL) = (20.2×O.D645) + (8.2×O.D663)
Carotenoid (µg/mL) = 4×O.D450
Lycopene (µg/mL) = 3.12×O.D502

2.2.7 Antioxidant enzymes measurements

2.2.7.1 Crude enzyme extract

Two grams of fresh leaves and stem were homogenized separately with 20 mM Tris–HCl buffer, pH 7.2 using homogenizer. The homogenates were centrifuged at 13000 rpm for 10 min at 4℃. The supernatants were stored as crude extract at -20℃ for further analysis.

2.2.7.2 Peroxidase assay

Peroxidase activity was carried out according to Miranda et al. [22], the reaction mixture was prepared by mixing 1 mL (40 mM guaiacol, 8 mM H_2O_2, 50 mM sodium acetate buffer, pH 5.5) and 0.1 mL extract. The change in absorbance at 470 nm due to guaiacol oxidation was recorded for 1 min using a spectrophotometer. One unit of peroxidase activity was calculated as the amount of enzyme which increases the O.D. 1 per min under standard assay conditions.

2.2.7.3 Polyphenol oxidase assay

Polyphenol oxidase activity was assayed with catechol as a substrate according to the spectrophotometric procedure of Jiang et al. [23]. 0.2 mL crude extract was added to 2.8 mL of 20 mM catechol reagent prepared in 10 mM PBS, pH 6.8. The absorbance at 400 nm is recorded for 3 min using a spectrophotometer. 1 unit of enzyme activity is calculated as it causes a change of 0.1 in absorbance per min under standard assay conditions.

2.2.7.4 Catalase assay

Catalase activity was determined according to Bergmeyer et al. [24]. 2 mL of substrate solution was made up of 25 mM H_2O_2 in a 75 mM PBS pH 7.0 with 0.5mL crude extract. The absorbance at 240 nm was recorded for 1 min using a spectrophotometer. 1 unit of enzyme activity was calculated as mentioned above.

2.3 Statistical Analysis

The statistical analyses were performed by the Student's t-test. The results were expressed as means ± SD (n = 3). The difference was considered significant when $P < 0.05$.

3. RESULTS

3.1 Antiglycation Assay Results

As it shown in Fig. 1. Extracts from leaves exhibited strong antiglycation activity than extracts from stem while both extracts significantly decreased AGEs formation.

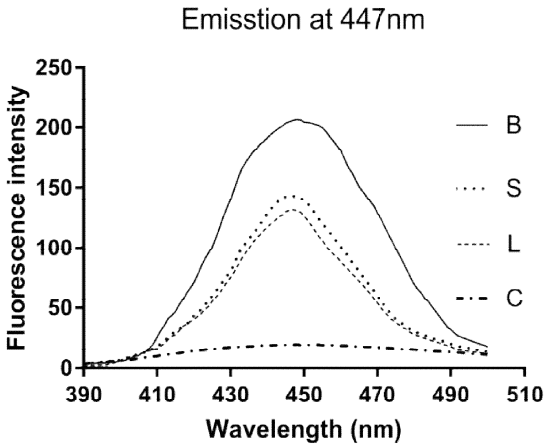

Fig. 1. b - bovine serum albumin with glucose (Group B), c - bovine serum albumin (Group C), s - bovine serum albumin with glucose and stem extract (Group S), l- bovine serum albumin with glucose and leaves extract (Group L).

p<0.05 for s to b and l to b
Mean ± S.E.M = Mean values ± Standard error of means of three experiments

3.2 Glucose Trapping Assay Results

Glucose concentration was estimated on 1th, 7th and 15th day of reaction. Fig. 2 showed that both extracts significantly reduced glucose concentration in the reaction system, compared to the control group. Leaves extracts showed stronger ability to trap the glucose than stem extracts.

3.3 0.5% MPBA Gel Results

Modified BSA was analyzed by special 0.5% MPBA gel due to its affinity. From Fig. 3, it can be visualized that in lane B, there is more modified BSA compared to lane C. Decreased BSA modification was detected in lane S and lane L which represents stem extract and leaves extract.

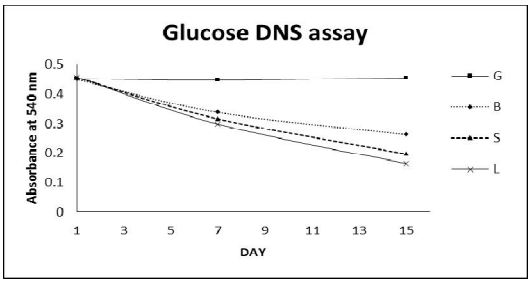

Fig. 2. B-bovine serum albumin with glucose (Group B), G-glucose (Group A), S- bovine serum albumin with glucose and stem extract (Group S), L-bovine serum albumin with glucose and leaves extract (Group L).

p<0.05 for S to B and L to B
Mean ± SD = Mean values ± Standard deviation of means of three experiments

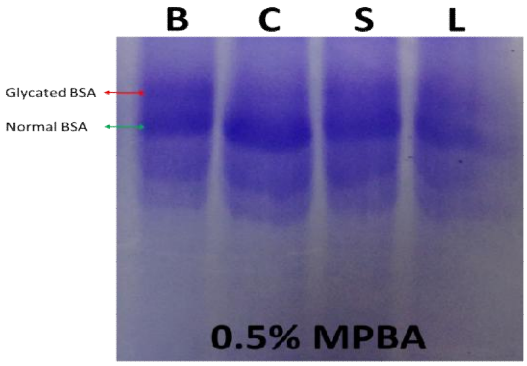

Fig. 3. B lane- bovine serum albumin with glucose(Group B), C lane- bovine serum albumin (Group C), S- bovine serum albumin with glucose and stem extract (Group S), L-bovine serum albumin with glucose and leaves extract (Group L).

3.4 Estimation of Non-enzymatic Antioxidant Components

Table 1 exhibited the non-enzymatic antioxidant components of both leaves and stem. All the estimated components are higher in leaves than in stem.

3.5 Antioxidant Activity Results

For evaluation of the antioxidant capacity of extracted compounds, commonly 4 different types of assays are conducted, and in this study, DPPH, ABTS and FRAP assay are tested. Fig. 4 illustrated that both extracts showed dose-dependent DPPH scavenging activity with IC_{50} 7.6 ug/mL for leaves extract and 22.3 ug/mL for stem extract. ABTS test result is given in Fig. 5, the leaves extract showed more scavenging activity with less amount (IC_{50} = 1.16 ug/mL), however, stem extract scavenged 50% ABTS with concentration 5.02 ug/mL. in FRAP assay more extracts were consumed to reduce 50% free radicals. The IC_{50} values are 14.6 ug/mL and 96.15 ug/mL respectively for leaves and stem extracts.

3.6 The Antioxidant Enzyme Activities

The level of enzymatic antioxidants such as, Peroxidase, catalase, and Polyphenol oxidase values were showed in Table 2. Peroxidase were found to be 19.3 ± 0.023 units/g tissue and 51 ± 0.032 units/g tissue for leaves and stem respectively. Polyphenol oxidase level was found to be the highest (1226.7 ± 0.016 units/g tissue) in leaves and 106.9 ± 0.021units/g tissue in stem. Catalase activity was found to be 496.6 ± 0.032 unit/g tissue in leaves and 413.2 ± 0.012 unit/g tissue in stem.

4. DISCUSSION

The using of plants in Saudi Arabia for the cure of many ailments is ancient and still available among the tribal and local people and medicinal healers. Therefore, the present study was conducted to study the antiglycation and antioxidant properties of stem and leaves of the medicinal plant, *C. forskohlii* Balady. The anti-glycation assays resulted in decreased AGEs level. This might be the extracts glucose trapping potency as it was proved by results in Fig. 2. Xiaoming Li et al. [25] reported that Quercetin inhibited AGEs formation by trapping reducing sugars produced Quercetin-derivatives. In this study, 10.1 ± 0.011 (mg GAE /g tissues) phenolic components detected in leaves which higher than the stem. The activities of non-enzymatic antioxidants of stem and leaves of *C. forskohlii* are presented in Table 1. Recent studies have shown that, many phenolic content and related components contribute significantly to the total antioxidant activity of many plants [26]. The total flavonoid in the leaves (1.6 mg catechin equivalents/g dry tissue) was found to be significantly higher than in the stem (0.55 mg catechin equivalents/g dry tissue). Most of the natural antioxidants possess multiple functions. Therefore, a reliable antioxidant evaluation protocol requires different antioxidant activity assessments to take into account various mechanisms of antioxidant action [27]. Therefore, the evaluation of antioxidant activity of

methanolic extract of *C. forskohlii* was conducted by several methods. Scavenging the stable DPPH radical model is a widely used method to evaluate antioxidant activity. The degree of discoloration indicates the scavenging potential of the antioxidant extract, which is due to the hydrogen donating ability [28]. The *C. forskohlii* extract showed a concentration-dependent scavenging of DPPH radical, which may be attributed to its hydrogen donating ability. The correlation coefficient (R^2) between the phenolic concentration of leaves, stem and DPPH scavenging activity was found to be 0.946 and 0.979, respectively indicated the strong correlation. The ABTS assay is based on the ability of an antioxidant to scavenge ABTS radicals. It is a simple and commonly used method for the evaluation of antioxidant capacity [12]. In this study, the correlation coefficient (R^2) between the phenolic concentration of leaves, stem and ABTS scavenging activity was found to be 0.988 and 0.995 indicating a high correlation. Fig. 6 shows the reducing power of the leaves and stem of *C. forskohlii*. The presence of reducers triggers the conversion of the Fe3+ /ferricyanide complex to the ferrous form. The formation of Perl's Prussian blue detected at 700 nm indicates a higher reducing power. The reducing power of stem (EC50 96.15 µg phenolic concentration/mL crude methanol extract) was found to be significantly higher ($P < 0.05$) than leaves (EC50 14.6 µg/mL). The correlation coefficient (R^2) between the phenolic concentration of leaves, stem and the formation of the ferrous complex was found to be 0.996 and 0.974, respectively indicating the strong correlation. It was reported that the reducing properties are due to the presence of reductones. It exerts antioxidant action by breaking the free radical chain by donating a hydrogen atom [29].

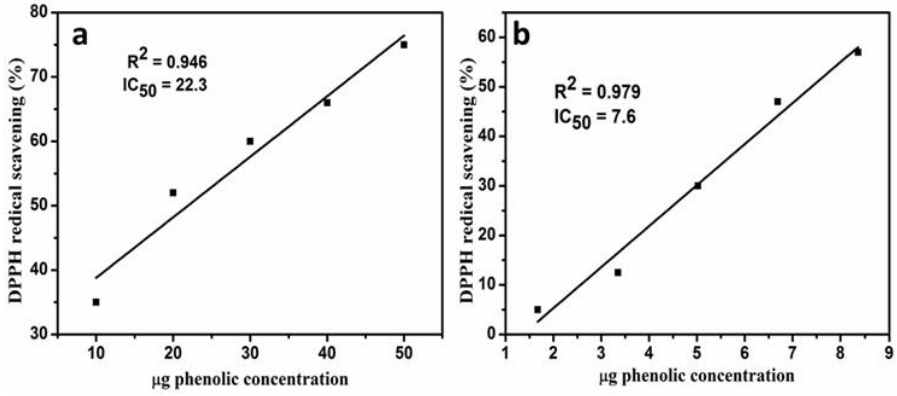

Fig. 4. Correlation between concentrations of phenolic compounds of leaves (a) and stem (b), and their antioxidant activity as determined by DPPH assay

Results are the mean ±SD, n=3.

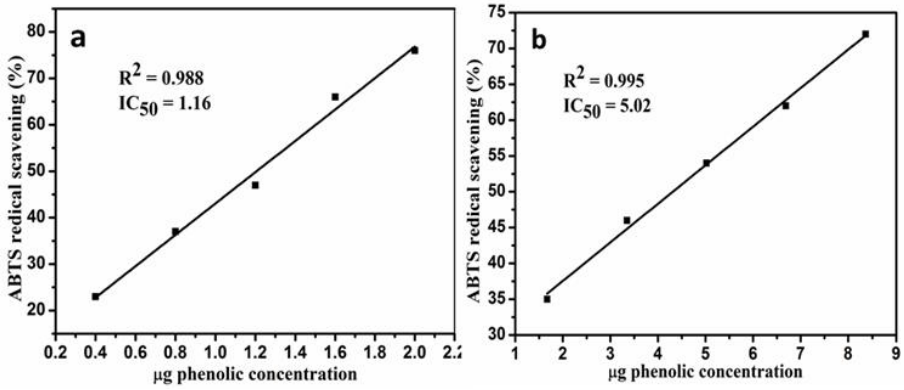

Fig. 5. Correlation between concentrations of phenolic compounds of leaves (a) and stem (b), and their antioxidant activity as determined by ABTS assay

Results are the mean ±SD, n=3.

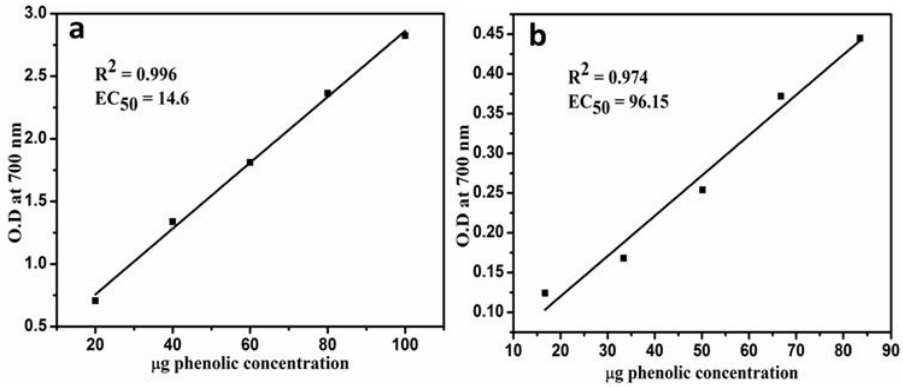

Fig. 6. Correlation between concentrations of phenolic compounds of leaves (a) and stem (b), and their antioxidant activity as determined by reducing power assay
Results are the mean ±SD, n=3.

Table 1. The total non-enzymatic antioxidants of *C. forskohlii*

Parameter	Stem	Leaves
Total phenolic content (mg GAE /g tissues)	2.5 ± 0.024	10.1 ± 0.011
Flavonoid content (mg CE/g tissue)	0.55 ± .031	1.6 ± 0.015
Chlorophyll (µg/ mL)	1.87 ± 0.021	2.82 ± 0.020
Lycopene (µg/ mL)	0.20 ± 0.034	0.30 ± 0.016
Total carotenoids (µg/ mL)	0.5 ± 0.033	0.61 ± 0.024

** Results are the mean ±SD, n=3.*

Table 2. The antioxidant enzyme activities of *C. forskohlii*

C. forskohlii	Peroxidase units/g tissues	Polyhenoloxidase units/g tissues	Catalase units/g tissues
leaves	19.3 ± 0.023	1226.7 ± 0.016	496.6 ± 0.032
stem	51 ± 0.032	106.9 ± 0.021	413.2 ± 0.012

** Results are the mean ±SD, n=3.*

The results for the Chlorophyll, lycopene and total carotenoid of the *Coleus forskohlii* (leaves and stem) are given in Table 1. Chlorophyll (2.82 µg/ mL), lycopene (0.30 µg/ mL and total carotenoid (0.61 µg/ mL) of the leaves were significantly higher ($P < 0.05$) than stem. The high amount of Chlorophyll, lycopene, and carotenoid were reported for medicinal plant *Coleus forskohlii* Briq (37.73 mg/g DW, 11.36 and 11.52 µg /g DW, respectively) [30]. Crude extracts of the various parts (leaves, fruits, roots, stem and trunk bark) of Garcina atroviridis showed strongest antioxidant activity than the standard vitamin E [31]. Aqueous extracts from the different parts of Momordica charantia, Glycyrrhiza glabra, Acacia catechu and Terminalia chebula were reported to be rich sources of enzymatic and non-enzymatic antioxidants [32].

The activities of the enzymes in the two parts of *C. forskohlii* are presented in Table 2. Polyphenol oxidase activity was found to be significantly higher in leaves (122.7 units/g tissue) than in stem (106.9 units/g tissue). Peroxidase activity was also found to be significantly higher in the stem (51 units/g tissue) than in the leaves (19.3 units/g tissue. Catalase activity too remained significantly higher in leaves (496.6 units/g tissue) than in stem (413.2 units/g tissue). A Caribbean copper plant peroxidase from the latex of *Euphorbia cotinifolia* was studied [33]. Catalase and antioxidant activity were screened in nine medicinal plants traditionally used in Chinese medicine [34]. A partial characterization of polyphenol oxidase activity of herb *Thymus longicaulis subsp. chaubardii var. chaubardii* is described [35]. Enzymatic antioxidants are an intrinsic defense tool to resist oxidative damage

in plants [36]. Results showed that *C. forskohlii* is reach source of enzymatic antioxidants.

5. CONCLUSION

The process of protein glycation impairs the biological activity of proteins and triggers their degradation process. The inhibition of glycation reaction could protect proteins from AGEs formation and could be one of the alternative therapy to avoid diabetic complications. Various compounds of natural such as the flavonoid, phenol derivatives have shown significant inhibition of the protein glycation and formation of AGEs. The mechanisms may involve the trapping of excessive reactive glucose, making them unable to react with proteins amino groups. Thus, antiglycation therapy will be an effective strategy in future to prevent the formation of AGEs for the management of late diabetic complications. The leaves possessed the significant potential of both non-enzymatic and enzymatic antioxidants that could be used against oxidant and free radical damages. Thus, the *C. forskohlii Balady* could be employed in all medicinal preparations to combat various diseases associated with glycation, oxidative stress, including diabetes and related disorders.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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