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Preliminary Phytochemical Analysis and *in vitro* antioxidant Potential of Fruit Stalk of *Capsicum annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill

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Abstract

The aim of the present study was to assess and evaluate the therapeutic value of fruit stalk of *Capsicum annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill found across homegardens of Uttar Kannada. The study involved the screening of phytochemical (qualitative) and quantitative estimation of phenolic content, flavonoid content and to assess their antioxidant potential. Antioxidant activity was evaluated using different *in vitro* assays such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), reducing power assay and total antioxidant capacity. The study indicated presence of different phytochemicals and constituted fair amount of phenolics and flavonoids, and exhibited antioxidant activity in a dose dependent manner. Thus, the present study presents a comprehensive knowledge on the therapeutic value of *C. annuum* var. *glabriusculum* fruit stalk which is commonly utilized for medicinal purpose across homegardens of Uttara Kannada.

Keywords: Homegardens, Phenolic content, Phytochemicals, Antioxidant potential, Medicinal plants.

INTRODUCTION

Plants are basic providers of food, fodder and fuel. Apart from these inherent properties, plants also act as reservoir of medicines that are found in the form of secondary metabolites. Till date several potential phytochemicals have been isolated and the structure has been elucidated from wide array of plants. Based on their bioactivity they are often categorized as medicinal plants and non-medicinal plants. A great number of plants have been exploited in search of phytochemicals. Extensive studies have been reported in assessment of antimicrobial and antioxidant activities [1]. However the search for novel phytochemicals is still in progress for the increasing incidence of diseases due to ever increasing population. Phytochemicals have received a great deal of attention due to the very reason that they are non-toxic and often show any side effects [2]. Phytochemicals as a whole is a vast group and generally comprise phenolics, tannins, flavonoids, alkaloids, saponins and carotenoids to name a few [3]. Among these classes of compounds phenolics is the most extensively studied group wherein several bioactivities have been attributed to it including antimicrobial, antioxidant, anti-inflammatory, anti-diabetic, anti-hypertensive, anticancer and many more [4].

Excessive free radicals or reactive oxygen species (ROS) generated in the body result in oxidative stress which is responsible for multiple chronic diseases including neurodegenerative diseases and cardiovascular diseases [5]. Several studies have hypothesized that secondary metabolites of plants are able to scavenge free radicals naturally [6]. Phenolics and non-phenolic compounds such as vitamins play a vital role in inhibiting oxidative stress. Several potential phenolic compounds that are widely distributed across plants act as antioxidants. Numerous antioxidants have been identified and isolated from various parts of plant. Nevertheless some antioxidants are chemically synthesized such as Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) Propyl gallate (PG) and Tert-butylhydroquinone (TBHQ) that are known to cause several severe side effects. As a result the quest for search of potential natural antioxidants has been the main goal of phytochemists.

Homegardens serve to be primary source of medicine for wide array of diseases across Western Ghats. Traditionally, several plant species of homegardens are used for treatment of health ailments [7, 8]. Locally, *C. annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill is used as coolant and to cure ulcer. Flavonoids and antioxidant activity of *Capsicum annuum* cultivars has been well documented [9, 10]. Peppers are found to comprise good quantity of flavonoids especially Quercetin and luteolin. Though *Capsicum annuum* has been highly domesticated as a spice, Ethanopharmacological studies have shown that Capsicum species are effective medicines against several health ailments including arthritis, rheumatism, snake bite and wounds [11]. However, understanding its medicinal value is relatively essential as studies have proved that geographical variations/environmental factors have impact. Hence in the present study an attempt was made to evaluate the therapeutic value of fruit stalk of *C. annuum* var. *glabriusculum* commonly grown across homegardens of Uttara Kannada.

MATERIAL AND METHODS

Chemicals

Gallic acid, quercetin, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1- picrylhydrazyl hydrate (DPPH) were purchased from Sigma Aldrich, Bangalore. All other chemicals used were of analytical grade and purchased from Merck, Bangalore.

Collection of plant material and identification

The plant was collected between May to September across homegardens of Uttara-Kannada, Karnataka, India. The Botanical name was authenticated using multiple floras at the Department of Botany, Government Arts and Science College Uttara Kannada, Karnataka, India. The ethnobotanical information of the plants considered for the present study is listed in Table 1.

Solvent extraction

The plant sample was shade dried at room temperature (31±4 °C) for 21 days, further they were homogenized into fine powder with the aid of electric grinder (TTK Prestige, India). The methanolic extract was prepared by macerating 10 g of plant sample in 100 ml of methanol for duration of 48 h with constant shaking using a rotary shaker (Remi, India). The crude extract was filtered through a Whatmann filter paper No. 1. Further the excess solvent was removed using a rota-evaporator (Ika, Bangalore, India). Finally filtrate was evaporated to dryness in a water bath at 60 °C. A known concentration of 10mg/ml was prepared which was stored at -20°C for further analysis.

Qualitative phytochemical analysis

The crude methanolic extract was subjected for qualitative phytochemical screening following the protocols as described by Harborne [12].

Quantification of total phenolic content

The total phenolic content (TPC) in the methanolic plant extract was estimated by following Folin-Ciocalteau's (FC) method as previously reported by Kumar et al [13]. Different aliquots of plant extracts and standards (1mg/ml) were taken in series of test tubes and volume was made up to 3 ml by adding deionized water. FC reagent (0.5ml) was added to each tube which was incubated for 3 min then sodium carbonate (20%, 2ml) was added, vortexed and further these tubes were kept in boiling water bath for 1 min. Absorbance was recorded at 650 nm using a double beam UV-Visible Spectrophotometer (U-2900, Hitachi, Japan) against reagent blank. Gallic acid was used as a reference standard and calibration curve was plotted (20-100 µg/ml) and TPC in plant extracts were expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/g dw).

Quantification of total flavonoid content

Total flavonoid content (TFC) of the methanol extracts was determined following the protocol described by Helmja et al [14], with slight modifications. Briefly, Known aliquots of extracts (500 µg/ml) and various concentrations of standards were pipetted out in a series of test tubes and volume was made up to 5 ml with deionized water. Sodium nitrite (5%; 0.3 ml) was added which was incubated for 5 min to this aluminium chloride solution (10%; 0.6 ml) was added with further incubation for 5 min, and, sodium hydroxide solution (1 M, 2 ml) was added and total volume was made up to 1 ml with distilled water. Absorbance was recorded at 510 nm against blank using a double beam UV-Visible spectrophotometer (U-2900, Hitachi, Japan). A standard curve was plotted using various concentrations of Quercetin (20-100 µg/ml). From the standard curve, concentration of flavonoids in the crude extracts were determined and expressed as milligrams of Quercetin equivalents per gram dry weight (mg QE/g dw).

In-vitro antioxidant assays

DPPH radical scavenging activity

DPPH free radical scavenging activity determined spectrophotometrically following the protocol of Blois [15] and as reported by Kumar et al [16]. Briefly, 0.2mM of DPPH solution was mixed to various concentrations of sample (1-5 mg/ml), vortexed and incubated for 30 min at 37±2°C in dark absorbance was read at using a double beam UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan). Methanol with no added samples was taken as control. Percentage radical scavenging activity (RSA) was calculated using the formula:

% RSA = $(A_{control}-A_{sample}/ A_{control}) \times 100$, where, A=absorbance at 517 nm.

ABTS assay

ABTS assay was performed as per the procedure described by Re et al [17]. Briefly, 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution was prepared. Working solution was prepared by mixing these two stock solutions in equal volume and reaction mixture was kept for incubation in dark for overnight at room temperature. 1 ml reacted ABTS solution was diluted with 60 ml of methanol to attain an absorbance of 1.170±0.02 units at 734 nm using a UV-Visible Spectrophotometer (UV-1800, Shimadzu, Japan). A 300 µl of different concentrations (200-1000 µg/ml) of crude methanolic extracts were mixed with 2700 µl of the reacted ABTS solution, which was allowed to react in dark. The absorbance was recorded at 734 nm.

Total antioxidant capacity

Total antioxidant activity of the plant extracts was determined following protocol of Prieto et al [18] with slight variations in reaction volume. 0.2 ml of crude extracts of various concentrations (12.5, 25, 50, 100 µg/ml) were taken in a test tube to which 1.8 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The tubes were incubated at 95°C for 90 min in a water bath and allowed to cool. The absorbance was measured at 695 nm against a blank using double beam UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The values are represented as µg ascorbic acid equivalents per gram dry weight (µgAAE/g dw).

Reducing power activity

Reducing power activity of test samples was determined following Phosphomolybdenum method as described by Oyaizu et al [19]. Briefly, Plant extracts of various concentrations 200-1000 µg/ml (made up to 1.0 ml with methanol) was taken in a series of test tubes to which 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) was added, and allowed to stand at 50°C for 20 min in a water bath. Further 2.5 ml of

10% trichloroacetic acid was added, which was centrifuged at 650 rpm for 10 min. 2.5 ml of supernatant was taken out and mixed with 2.5 ml distilled water to which finally 0.5 ml of $FeCl_3$ (0.1%) was added. Absorbance was recorded at 700 nm against reagent blank using a double beam UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan).

Statistical Analysis

Data are represented as mean ± SD for triplicate determinations. Analysis of variance and Tukey's t-tests was done to identify differences among means using Graph prism statistical software. Statistical significance was declared at p = 0.05.

Table 1. List of plants and the parts used with their associated traditional knowledge

Botanical Name	*C. annuum* var. *glabriusculum*
Family	Solanaceae
Part used	Fruit stalk
Associated Traditional Knowledge	Used as coolant and to cure ulcer

DISCUSSION AND RESULTS

Now-a-days, phytochemicals analysis among plants are gaining importance due to the very reason that they possess multiple bioactivities including antioxidant properties which are used for treatment of wide array of chronic and infectious diseases. The phytochemical screening of the methanolic crude extracts of fruit stalk of *Capsicum annuum* revealed the presence of alkaloids, tannins, glycosides, phenolics and flavonoids. The result has been tabulated in table 2.

Table 2. Phytochemical analysis of methanolic crude extracts

Plant extracts	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
C. annuum var. *glabriusculum*	+	+	+	+	+	+	-	+	-	-	-

The sign (+) denotes the presence of the compound whereas (-) indicates absence of compound. A: Phenolics; B: Tannin; C: Flavonoids; D: Alkaloids; E: Glycosides. F: Terpenoids; G: Steroids; H: Saponins; I: Anthraquinone; J: Phlobatanin; K: Oils and Fats.

Total phenolic content (TPC) and Total flavonoid content (TFC) and percentage extraction yield of *Capsicum annuum* methanolic extract is tabulated in Table 3, The FC method is widely accepted technique used to quantify the phenolics which is manly dependent on the reduction of metal oxides. The reduction leads to the formation of blue color complex which is read at absorbance of 650nm. Phenolics as secondary metabolites are the principal components of phytochemicals and have been attributed to the antioxidant potential of plants [20]. The TPC was calculated based on the gallic acid standard curve (Y = 0.0223X; R^2 = 0.9907). The fruit stalk of *Capsicum annuum* comprised phenolic content of 29.051± 0.21 mg GAE/gdw. TFC analysis among indicated concentration of 2.778±0.14 mg/GAE/g dw.

Table 3. TPC, TFC and extraction yield values of crude methanolic extracts of five plant species.

Plant extracts	**TPC** (mg GAE/g dw)	**TFC** (mg QE/g dw)	**Extraction yield** (%)
C. annuum var. *glabriusculum*	29.051± 0.21	2.778±0.14	24.97

Values represented as Mean±SD of triplicate determinations

In vitro antioxidant activities

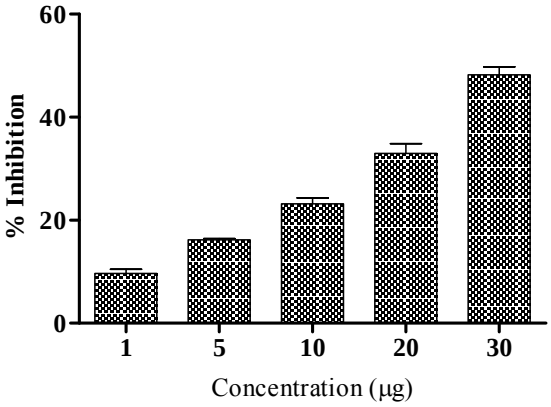

Figure 1: DPPH radical scavenging activity crude methanolic extracts of *C. annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill

DPPH is a fast, reliable, simple and most preferred method employed for evaluating the antioxidant potential of wide array of test samples. DPPH is a stable free radical since delocalization of spare electron occurs over the entire molecule. Proton donation by the the antioxidants present in test samples to the DPPH radicals reduces deep violet colour to colourless in the reaction mixture [21, 22]. Figure 1, highlights percentage radical scavenging activity exhibited by various concentrations of plant extracts. In the present study it was observed that *C. annuum* var. *glabriusculum* exhibited highest DPPH radical scavenging activity. A recent study by Gurnani et al. [23] on *Capsicum annuum* reported that n-hexane (26.9%) and chloroform (30.9%) seed extracts demonstrated DPPH radical scavenging activity at the concentration of 1 mg/ml. wherein in the present study *C. annuum* var. *glabriusculum* methanolic extract of fruit stalk exhibited 26.05 % against DPPH free radical at a concentration of 1mg/ml. The inhibition percentage showed a significant increase corresponding to increased concentrations of plant extracts. Several previous studies have reported significant correlation between TPC and antioxidant activity. However, in the present study it was observed that TPC and DPPH free radical scavenging activity of crude methanolic extracts of five plant species demonstrated significant correlation with a Pearson's coefficient value of 0.947.

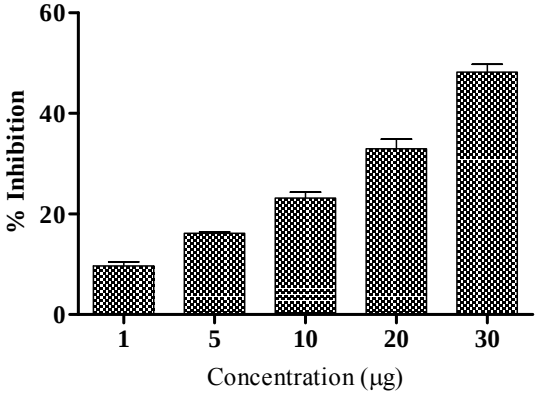

Figure 2: ABTS radical scavenging activity of *Capsicum annuum* (fruit stalk) crude methanolic extract

iABTS assay is one of the established and most followed *in vitro* antioxidant evaluation techniques. Several previous studies have highlighted the efficiency of ABTS free radical scavenging assay which is very sensitive when compared to other antioxidant assays. Figure 2 depicts the ABTS radical scavenging activity of *C. annuum* var. *glabriusculum* crude methanolic extract was 451.03±3.00 µg/ml. In addition, the correlation analysis between the TPC and ABTS assay indicated positive correlation with a Pearson's coefficient value of 0.968 among all the tested plant species.

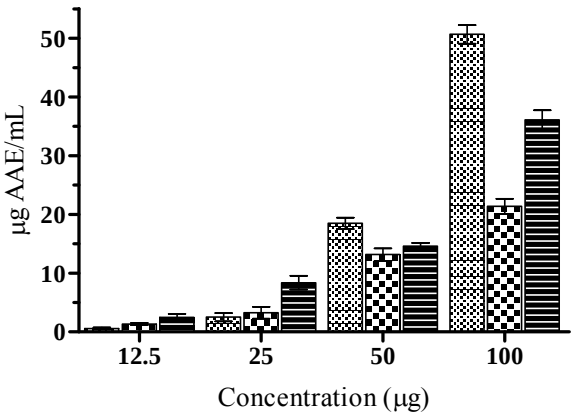

Figure 3: Antioxidant capacity of crude methanolic extracts of *C. annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill expressed as ascorbic acid equivalents (µg/g dw) by phosphomolybdenum method.

TAC was analyzed by phosphomolybdenum method. Generally, it involves reduction of Mo(VI) to Mo(V) by the test sample that results in formation of green colour complex. *C. annuum* var. *glabriusculum* methanolic extract exhibited TAC of 1304.23±21.98 µgAAE/g dw However according to previous reports numerous mechanisms including inhibition of chain initiation, binding of transition metal ion catalysts, and free radical scavenging are responsible for antioxidant activity [24].

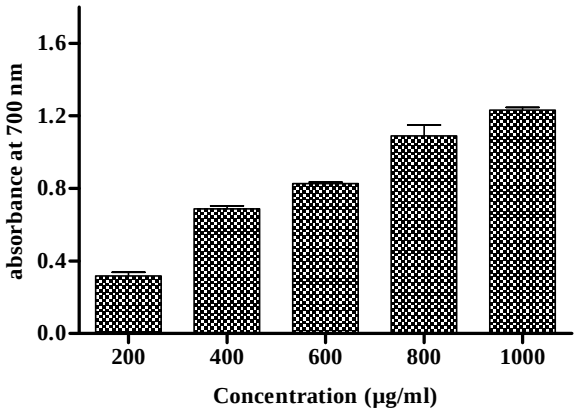

Figure 4: Reducing power assay of crude methanolic extracts of *C. annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill

Reducing power assay is one of established method for evaluation of antioxidant potential of a test sample. Basically, it involves reduction of Fe3+ into Fe2+ with the formation of Perl's Prussian blue colour complex wherein absorbance is read at 700 nm [25]. This reducing ability varies with respect to various concentrations of antioxidant present in the samples. Moreover, the reducing power ability mainly depends on the bioactive compounds including phenolics present in the test samples. Figure 4 highlights the reducing power activity of *C. annuum* var. *glabriusculum* methanolic extract of fruit stalk.

However, according to literature *Capsicum* species have been largely studied because of its commercial and medicinal values. Multiple bioactive compounds have been elucidated from variety of Capsicum species such as capsaicin and capsaicinoids [26]. A recent study of Zimmer et al [11] reported the phenolic and flavonoid contents of fruit and seeds of *Capsicum baccatum*, which was found to exhibit antioxidant activity in a dose dependent manner.

Further, it should be noted that antioxidant potentialities showed a great variations against four antioxidant systems assayed, similar observations were reported earlier [27, 28]. However, in the present study, results of the TAC, ABTS, DPPH and RPA antioxidant assays indicated a significant difference between antioxidant potential of crude methanolic extract. This phenomenon could be attributed to the various antioxidants present in the plant extracts and their differences in chemical structure which enables donation of proton to the different free radicals. Further, it is also noteworthy that other than phenolics several other potent bioactive compounds such as vitamin C, E and volatile constituents present in the test samples also scavenge free radicals [29, 30]. Nevertheless, reports also suggest the antioxidant potential of the extracts may be attributed to synergistic effect involving both phenolics and other bioactive compounds [31, 32]

CONCLUSIONS

The results of the study revealed that *C. annuum* var. *glabriusculum* (fruit stalk) methanolic extract possess antioxidant properties which confirm its therapeutic nature. In addition, A significant positive correlation was observed between TPC and various antioxidant assays (DPPH and ABTS). Thus the study highlights preliminary evidence on therapeutic nature of *C. annuum* var. *glabriusculum* which is widely utilized across homegardens of Uttara Kannada. Further, fruit stalk of *C. annuum* var. *glabriusculum* may prove to be promising source of natural antioxidants. Nevertheless, extensive research is further essential for the identification and isolation of key bioactive constituents responsible for the antioxidant property.

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