

Full Length Research Paper

Antioxidant studies on the ethanolic extract of *Commiphora* spp.

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This investigation elucidated the presence of phytochemical constituents and *in vitro* free radicals scavenging activity for nitric oxide, total reducing power, superoxide, lipid peroxidation and DPPH in the ethanolic leaves extract of *Commiphora* species; *Commiphora caudata* (CC) and *Commiphora var pubescens* (CP). The IC₅₀ values of both the species were comparable to standard drugs, Quercetin (nitric oxide), vitamin C (superoxide), vitamin E (lipid peroxidation), vitamin C (DPPH). The results were analyzed statistically by regression analysis. In all the *in vitro* assays, the ethanolic extracts of the leaves showed its ability to scavenge free radicals in a dose dependent manner. However our study revealed that CC has potent antioxidant activity better than CP. Further investigation on the isolation, identification of antioxidant components in these plants may lead to chemical entities with the potential for clinical use and evaluation of *in vivo* antioxidant activity.

Key words: *Commiphora caudata*, *Commiphora var pubescens*, radical scavenging activity.

INTRODUCTION

Although oxygen is essential for aerobic process, cells under aerobic condition are threatened with the insult of reactive oxygen metabolites, a threat which is efficiently taken care of by the powerful antioxidant system in human body (Musa, 2008). Aerobic life is characterized as the continuous production of oxidants balanced by an equivalent synthesis of antioxidants (Yaushisakono, 1978). The improper balance between reactive oxygen metabolite production and antioxidant defense results in "oxidative stress" which deregulates the cellular function leading to various pathological conditions (Raja Sudarajan et al., 2006). Antioxidant principles from natural sources possess multifacetedness in their multitude and magnitude of activities to provide enormous scope in correcting the imbalance (Malencic et al., 2000). Ancient literature mentions many herbal medicines for treating various diseases like diabetic mellitus, rheumatoid arthritis and cardiovascular diseases. Unfortunately in India many potential medicinal plants used as ancient folklore medicine, lack scientific documentation. Two of such plants *Commiphora caudata* (CC) and *Commiphora*

var pubescens (CP) were selected to explore the potent bioactive compounds for its antioxidant activity to discover the actual value of folkloric remedies. *C. caudata* (Arn) Engl (Bursaceae) commonly known as "hill mango" is a moderate sized tree/shrub occurring in dry forest and commonly cultivated as an avenue tree (Nayar, 1956) found throughout South India. *C. var pubescens* (Wight & Arn) belongs to the same family a variety of *Commiphora* species, a middle sized tree/shrub (Gamble, 1997) which also grows in the dry forest of South India. Its gum resin from the bark is used for treating stomach troubles (Latha et al., 2005). Although reports on antibacterial and diuretic activity are available (Latha et al., 2005; Selvamani et al., 2004) but till now no experimental work has been carried out to verify the claims on the antioxidant status even though it was practiced as village folklore medicine for treating ulcer, inflammation, diarrhoea and spasms (Zang, 2000). Hence the present study focused on the *in vitro* antioxidant models for these species.

MATERIALS AND METHODS

Chemicals and instruments

All the chemicals and solvents used in this study was of analytical grade available commercially. DPPH (1,1-diphenyl-2-picrylhydrazyl), sodiumnitrite, potassium hexacyanoferrate

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were purchased from Sisco Research Laboratories Pvt .Ltd.,India. Nitro blue tetrazolium chloride, vitamin C, vitamin E and thiobarbituric acid were obtained from Himedia Laboratories Ltd., Mumbai, India. Rutin, Quercetin were obtained from Sigma Aldrich St Louis, USA. UV spectrometer (Shimadzu 160 IPC), homogenizer, centrifuge (Remi, India) and pH meter (Elico Ltd., India) were the instruments used for the study.

Plant material

The leaves of *C. caudata* and *C. var pubescens* were collected from Tirunelveli and Pudukotai districts of Tamil Nadu during the month of January 2008 and the plant material was identified by BSI (Botanical Survey of India, Coimbatore, Tamil Nadu, India).

Preparation of extract

The shade dried and powdered leaves of CC and CP was extracted by continuous hot extraction process using soxlet apparatus (Borosil, India) with ethanol by cold maceration. After extraction, the extracts were then concentrated under reduced pressure.

Phytochemical analysis

Preliminary qualitative analysis of ethanolic leaves extracts of CC and CP was performed by using specific reagent mentioned in the standard procedures (Edoega et al., 2005).

Total polyphenolic content

Appropriately diluted sample in 1 ml was mixed with 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thereafter, it was thoroughly mixed and placed in the dark for 1 h and the absorbance was measured at 765 nm using UV/Visible spectrophotometer. A gallic acid standard curve was obtained for concentrations (50, 100, 150, 200, 250 mg/ml) of ethanol for the calculation of polyphenolic content (Chandler et al., 1993).

Total flavonoid content

1 ml of sample was mixed with 3 ml of ethanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and it was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ml ethanol (Chang et al., 2002).

Nitric oxide scavenging activity

The different concentrations (100, 200, 400, 750, 1500, 2000 $\mu\text{g/ml}$) of test extract was taken in the volume of 200 μl . Nitric oxide production was initiated with the addition of 200 μl of phosphate buffer (0.1 M, pH 7.4) and 800 μl Sodium nitroprusside (10mM) and the reaction mixture was incubated at 25°C for 150 min. A control tube was also processed in the same way except for test extract ethanol was used. After the incubation time 1.2 ml of Griess reagent was added to the tubes and they were kept at room temperature for 30 min and the color developed, the optical density was read at 540 nm (Green et al., 1982).

Total reducing potential

The extract (0.75 ml) at various concentrations (100, 200, 400, 500, 1000, 1500 $\mu\text{g/ml}$) was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate ($\text{K}_3\text{Fe}(\text{CN})_6$) (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of Trichloroacetic acid (TCA) solution (10%) and then centrifuged at 800 g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride (FeCl_3) solution (0.1%, w/v) was added and kept at room temperature for 10 min. The absorbance was read at 700 nm, higher absorbance of the reaction mixture indicate greater reducing power (Oyaizu, 1986).

Super oxide radical scavenging activity

The reaction mixture consists of 100 μl riboflavin solution (20 μg), 200 μl EDTA solution (12 mM), 200 μl ethanol and 100 μl NBT (nitro-blue tetrazolium) solution (0.1 mg) and 100 μl of test substance at concentrations (300, 600, 900, 1200, 1500 $\mu\text{g/ml}$) were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer (50 mM). The absorbance of solution was measured at 540 nm using phosphate buffer as blank after illumination for 5 min. A control tube without the test substance but with ethanol served as control (Halliwell and Gutteridge, 1985; Korycka et al., 1978; Ravishanker et al., 2002).

Lipid peroxidation inhibition

The reaction mixture containing 0.5 ml of 10% rat liver homogenate in 0.1 M KCl (pH 7.4) and 0.5 ml of various concentrations (80, 60, 240, 320, 400, 500 $\mu\text{g/ml}$) of test compounds and 0.2 ml of FeSO_4 (15 mM) was incubated at 37°C for 30 min and it was again treated with 1.0 ml 10% TCA and 1 ml of 0.67% thiobarbituric acid. This mixture was heated in 90°C for 20 min. The pink colour developed was read at 532 nm. The control tube contains methanol instead of test sample (Okawa et al., 1979).

DPPH decoloration assay

To 1.0 ml of 100 μM DPPH solution in ethanol equal volume of test extracts in ethanol of different concentration (20, 40, 60, 80,100 $\mu\text{g/ml}$) was added and incubated in dark for 30 min. The change in coloration was observed in terms of absorbance using Spectrophotometer at 514 nm. 1.0 ml of methanol instead of test extract was added to the control tube (Havsteen, 1983).

Statistical analysis

All results are expressed as \pm S.E.M. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Different phytochemicals have various protective and therapeutic effects which are essential to prevent diseases and maintain a state of well being. Ethanolic extract of leaves of CC and CP were analyzed for its phytoconstituents. CC and CP contains carbohydrates, phytosterols, saponins, proteins, amino acids, gums, mucilage and flavonoids, terpenoids and glycosides (data not shown). It is well known that plant flavonoids and phenols in general, are the highly effective free radical

Table 1. Flavonoids and phenol contents in the studied plant extracts.

Plant species	Flavanoid (mg/ml)	Phenol (mg/ml)
<i>C. caudata</i>	8.21 ± 0.50	20.8 ± 0.2
<i>C. var pubescens</i>	2.82 ± 0.23	10.01 ± 0.5

Value are mean ± SD (n = 6).

Table 2. IC₅₀ values for *C. caudata*, *C. var pubescens* ethanolic extracts and different standards in different *in vitro* free radical scavenging methods.

Model	<i>C. caudata</i> IC ₅₀ value (µg/ml)	<i>C. var pubescens</i> IC ₅₀ value (µg/ml)	Standard IC ₅₀ value (µg/ml)
Nitric oxide	810.33 ± 0.02	450.04 ± 0.01	Quercetin 1510.65 ± 0.01
SOD scavenging	569.33 ± 0.01	310.61 ± 0.03	Vitamin C 840.20 ± 0.02
Lipid peroxidation	340.52 ± 0.03	180.90 ± 0.02	Vitamin E 390.12 ± 0.01
DPPH scavenging	20.51 ± 0.03	8.39 ± 0.01	Vitamin C 35.24 ± 0.02

Value are mean ± SD (n = 6).

scavengers and antioxidants. Polyphenol and flavonoids are used for the prevention and cure of various diseases which is mainly associated with free radicals (Havsteen, 1983). It is well known that plant phenolics, in general, are the highly effective free radical scavengers and antioxidants. The content of total phenols and flavonoids of CC was found to be much higher than CP as shown in Table 1 with gallic acid and quercetin as standards. These results indicate that a higher antioxidant activity of the CC extract compared to the CP extract may be correlatable to the phenolic and flavonoid content of the respective plant extract. A similar finding has been demonstrated by *Commiphora mukul* (Zang, 2000). Vitamin C and E acts as an antioxidant in biological systems and scavenge the free radicals thereby increase the antioxidant defense in the body. The effect of vitamin C and other putative antioxidants on biomarkers of oxidation have been studied in many pathological states that are thought to result, or result in oxidative stress and used as standard drugs for various *in vitro* scavenging studies (Riemersma et al., 2000). Nitric oxide is a potent pleiotropic mediator of physiological process such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilatations and antimicrobial and antitumour activities (Hagerman et al., 1998). CC highly inhibited nitric oxide in dose dependent manner (Table 2) (Figure 1) where quercetin was standard drug which resulted in significant IC₅₀ value. For the measurements of the reducing ability, the Fe³⁺ to Fe²⁺ transformation was investigated in the presence of CC and CP. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been

assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity (Gordan, 1990; Oyaizu, 1986; Okawa et al., 1979; Scoot et al., 1993; Selvamani et al., 2004; Yaushisakono, 1978; Havsteen, 1983; Yu et al., 2004; Yilidirim et al., 2000). Figure 2 depicts the reductive effect of CC, CP and the standard drug Rutin increased with increase in dosage. All the doses showed significantly higher activities, indicating that both species consists of hydrophilic polyphenolic compounds that cause the greater reducing power. Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Okawa et al., 1979). The superoxide anion radical scavenging activity of the ethanolic extracts from CC, CP and vitamin C increased markedly with the increase of concentrations. The half inhibition concentration (IC₅₀) of CC was significantly higher than CP from Table 2 (Figure 3). These results suggested that CC has important superoxide radical scavenging effect. Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through OH radical by Fentons reaction. Figure 4 shows that the CC extract highly inhibited FeSO₄ induced lipid peroxidation in rat mitochondria as a dose dependent manner (Table 2) than CP with vitamin E as standard. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the OH radical or the superoxide radicals or by changing the Fe³⁺/Fe²⁺ or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. DPPH is a free radical stable at room temperature which pro-

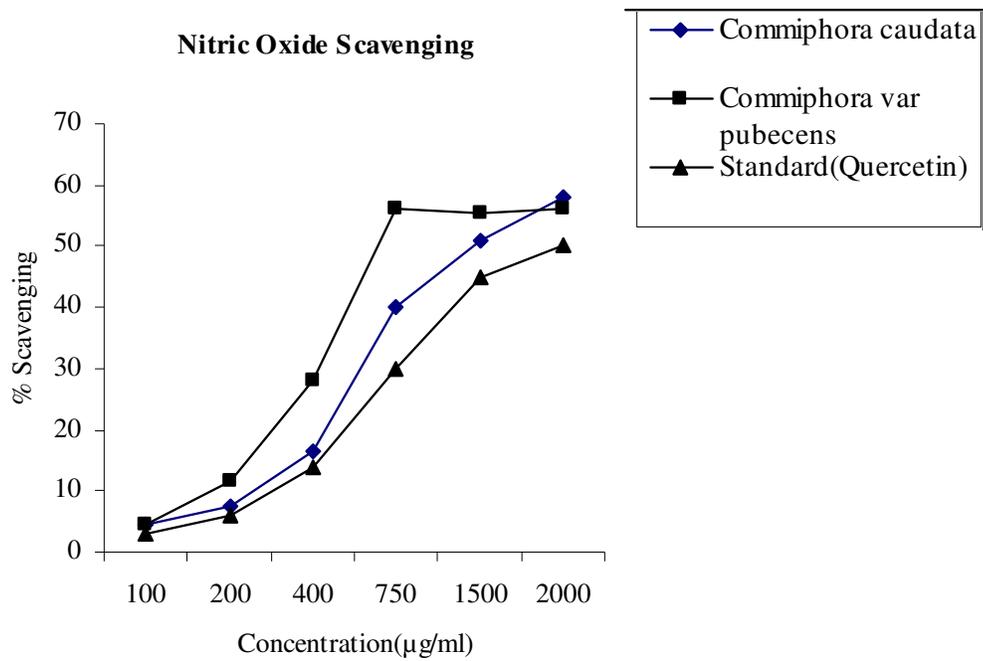


Figure 1. Nitric oxide scavenging activity of various concentrations of *C. caudate*, *C. var pubescens* and quercetin. The results are expressed in terms of concentration vs % scavenging. Each value represents mean value \pm SD of three experiments carried out each in triplicate samples.

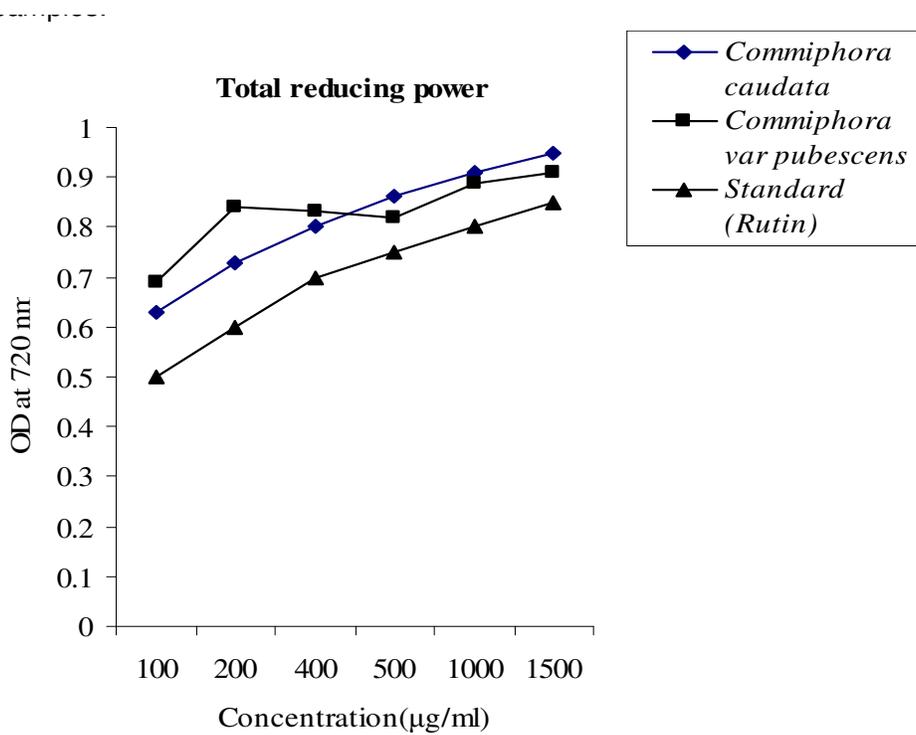


Figure 2. Total reducing power activity of various concentrations of *C. caudata*, *C. var pubescens* and Rutin. The results are expressed in terms of concentration vs OD at 720 nm. Each value represents mean value \pm SD of three experiments carried out each in triplicate samples.

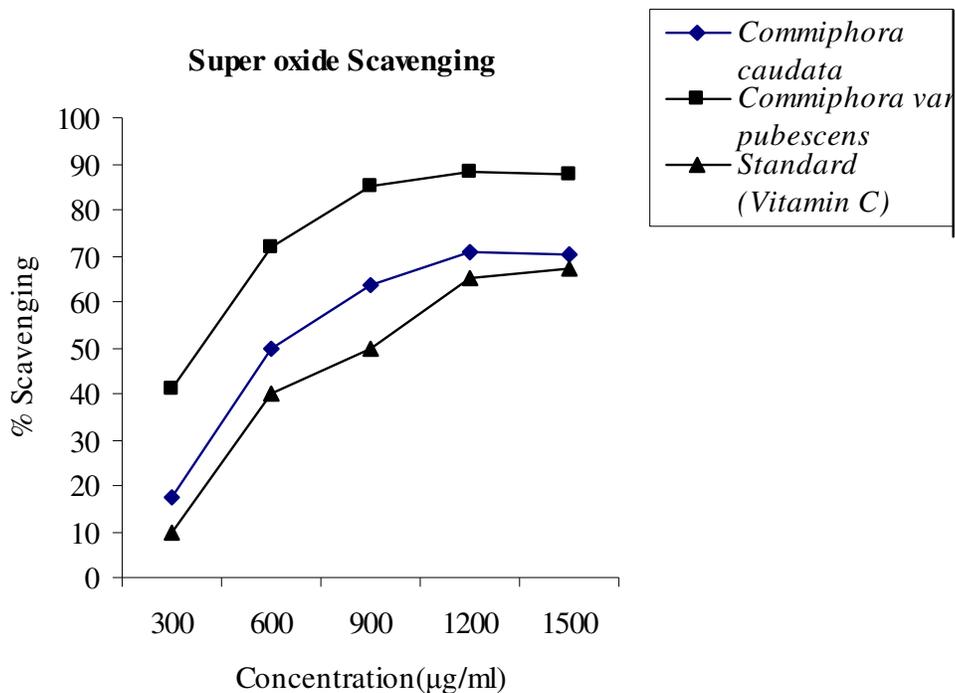


Figure 3. Super oxide scavenging activity of various concentrations of *C. caudata*, *C. var pubescens* and vitamin C. The results are expressed in terms of concentration vs % scavenging. Each value represents mean value \pm SD of three experiments carried out each in triplicate samples.

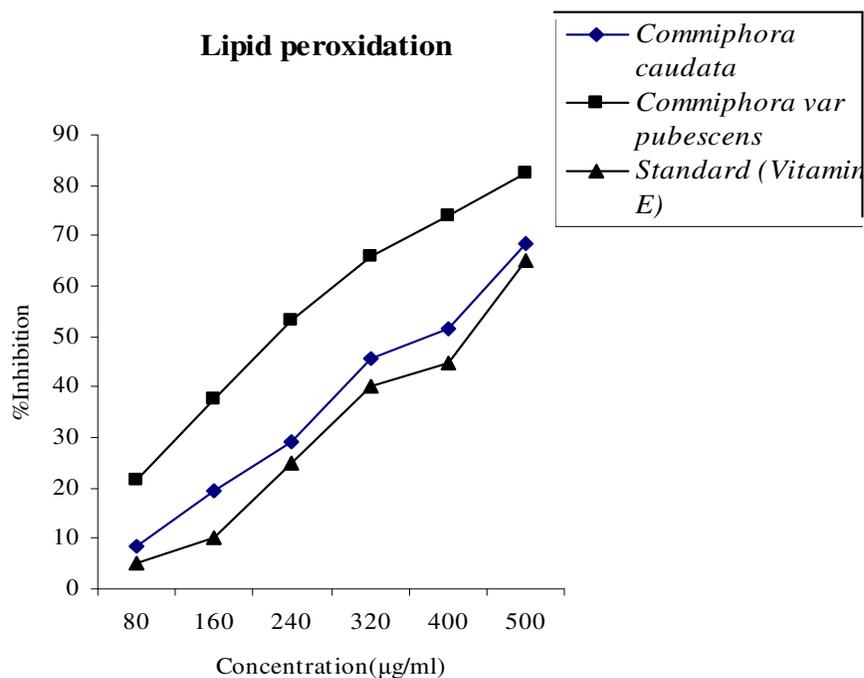


Figure 4. Lipid peroxidation activity of various concentrations of *C. caudata*, *C. var pubescens* and vitamin E. The results are expressed in terms of concentration vs % inhibition. Each value represents mean value \pm SD of three experiments carried out each in triplicate samples.

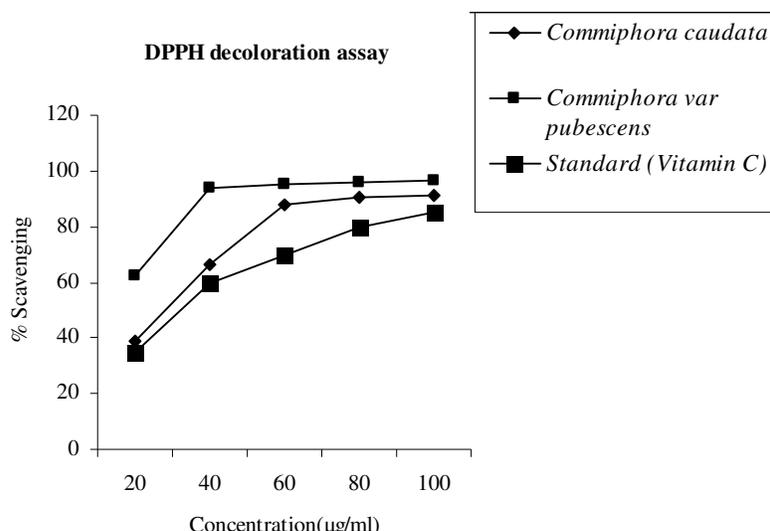


Figure 5. DPPH scavenging activity of various concentrations of *C. caudata*, *C. var pubescens* and vitamin C. The results are expressed in terms of concentration vs % scavenging. Each value represents mean value \pm SD of three experiments carried out each in triplicate samples.

duces purple colour solution in ethanol. It is reduced in the presence of an antioxidant molecule; giving rise to uncolored ethanol solution (Thabrew et al., 1998). Crude ethanolic extracts of CC exhibited a significant dose dependent inhibition of DPPH activity than CP (Table 2, Figure 5) where vitamin E is standard drug with much significant IC_{50} value. This indicates CC as a good source of natural antioxidants.

Conclusion

This study suggests that the extracts *C. caudata* possess more antioxidant activity than *C. var pubescens*, which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases. Further investigation on the isolation and identification of antioxidant components in this plant may lead to chemical entities with the potential for clinical use.

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