

Full Length Research Paper

## Bioassay-guided investigation of *Lonchocarpus cyanescens* benth leaves extracts for antioxidant activities

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Antioxidants have been reported to prevent oxidative damage caused by free radicals and can be used to ameliorate conditions in cardiovascular and inflammatory diseases. Ethnobotanical study revealed that the leaves of *Lonchocarpus cyanescens* are traditionally used in Africa to treat ulcer and arthritis. This study investigates the antioxidant activities of its extract and fractions. Acetone leaf extract of *L. cyanescens* was screened for 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing power (FRAP), total phenol content and total flavonoid content using catechin as standard antioxidant. Bioguided column chromatographic separation was carried out and the resultant fractions were screened for antioxidant activities. Preliminary spectroscopic profile of the most active fraction was obtained. DPPH and FRAP methods showed that *L. cyanescens* had antioxidant activity which correlated with its phenolic and flavonoid contents. There was a higher correlation of the total phenol/flavonoid content to the antioxidant activity by the DPPH method [ $r^2 = 0.9906, 9926$  respectively] than the FRAP method [ $0.8635, 8840$  respectively]. Bioactivity guided fractionation identified fraction F5 as the most active. <sup>1</sup>H and Infrared spectra indicated that the most active fraction contained flavonoids. Comprehensive *in-vivo* studies and toxicity profile of the extract will be required before considerations for development as a phyto-drug.

**Key words:** *Lonchocarpus cyanescens*, antioxidant, DPPH, FRAP, phenol, flavonoid.

### INTRODUCTION

Free radicals formed in the body are chemical species that possess an unpaired electron in the outer valance shell of the molecule. This makes them highly reactive and can complex with major macromolecules including proteins, lipids, carbohydrates and DNA (Evans and Halliwall, 1999). Free radicals may either be oxygen derived (reactive oxygen species, ROS) or nitrogen

derived (RNS, reactive nitrogen species) (Devasagayam and Kesavan, 2003). An imbalance between antioxidants and ROS results in oxidative stress (Mohammed et al., 2012). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, ulcer, arthritis and neurodegenerative disorders

(Peterhans, 1997; Ames et al., 1993).

Studies on medicinal plants and vegetables strongly supported this idea that plant constituents with anti-oxidant activity are capable of exerting protective effects against oxidative stress in biological systems and other health related benefits (Mali et al., 2004; Chaudhary et al., 2004). Recently, there has been growing interest in natural antioxidants of plant origin because they have greater application in the food industry for increasing the stability and shelf life of food products.

*Lonchocarpus cyanescens* (Schumach. and Thonn.) Benth is a shrub of twinning habit, belonging to the legumes family Fabaceae (Ogungbaro, 2010). Deciduous scandent shrub up to 4 m tall (in cultivation usually up to 2.5 m) or liana up to 20 m long; bark grey to very pale brown, slash yellowish; branchlets silky when young. It is widespread in Western Africa such as Cameroon, Ivory Coast, Sierra Leone, Benin, Nigeria, Togo and Guinea. It is also cultivated, particularly in Sierra Leone and Ghana. The plant is commonly known as West African indigo. The leaves of *L. cyanescens* is traditionally used for the treatment of diseases like arthritis, ulcer, intestinal disorder, dysentery, psychosis and leprosy (Ismot et al., 2013; Mubo et al., 2012; Iwu and Ayanwu, 1982). There is scanty scientific data to support the folkloric use of this plant in many of these disease states. The current study was designed to investigate the antioxidant activities of *L. cyanescens* using *in vitro* assay models which could possibly suggest the pharmacological basis of some of its local applications.

## MATERIALS AND METHODS

### General methods

Nuclear magnetic resonance (NMR) data were obtained on a Bruker 500 MHz model using deuterated methanol as solvent and tetra-methyl silane [TMS] as internal standard. Infrared data was obtained from a Bruker Fourier transform infrared (FT-IR) spectrophotometer model Vector 22. Silica gel 60 - 120 mesh was used for the Bioguided separation of bioactive extract. All Spectral data were obtained from the Facilities of Indian Institute of Integrative Medicine, Jammu, India.

### Plant material

Fresh leaves of *L. cyanescens* were identified and collected from the University of Ibadan Botanical Garden by Mr. M. K. Owolabi of the Department of Botany (Curator of the Botanical garden of the University of Ibadan). The leaves were air dried and milled into the

University of Ibadan). The leaves were air dried and milled into fine powders and kept in airtight containers until use.

### Extraction of plant

Powdered plant material (350 g) was extracted with 3.5 L of acetone by cold maceration with intermittent shaking for a period of 72 h. The extract was filtered through Whatman No.1 filter paper and concentrated to a minimum volume of solvent at 45°C with rotary evaporator. The extract was finally concentrated under a stream of cold air to dryness. The weight of the dried extract was determined and then stored in the refrigerator until use.

### Thin layer chromatography (TLC) analysis of extracts

The extracts were analyzed using TLC F254 with the following solvent systems: hexane: ethyl acetate (HE) (7:3), chloroform: methanol (CM) (9:1) and chloroform: ethyl acetate: formic acid (CEF) (5:4:1). Compounds were visualized by spraying with vanillin-sulphuric acid spray reagent (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid). The plates were heated at 110°C for 5 min to view the spots.

### Antioxidant assays

#### 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

Free radical scavenging activity was evaluated using catechin as standard antioxidant. The radical scavenging activity was measured using the stable radical DPPH according to the method described by Hatano et al. (1988). Various concentrations of the extracts were added to 4 ml of methanol solution of DPPH (1 mM, 1 ml). The mixture was shaken and left for 30 min at room temperature in the dark and the absorbance was measured with a spectrophotometer at 517 nm. All determinations were performed in triplicate. The antioxidant activity was calculated as the percent inhibition caused by the hydrogen donor activity of each sample according to the following:

$$\text{DPPH scavenged (\%)} = \frac{\text{A control} - \text{A test}}{\text{A control}} \times 100$$

Where, A control = absorbance of control and A test = absorbance of test sample.

#### Ferric reducing power (FRAP) assay

This was determined according to the method of Oyaizu (1986). The extract or standard (10-1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (pH 6.6) and potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 2.5 ml) was added to the mixture. A portion of the resulting

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**Abbreviations:** DPPH, 2,2-Diphenylpicrylhydrazyl; FRAP, ferric reducing power.

**Table 1.** Antioxidant activity of *L. cyanescens* extract by DPPH method.

Concentration ( $\mu\text{g/ml}$ )	% Antioxidant activity	
	<i>L. cyanescens</i>	Catechin
500	14.83 $\pm$ 0.386	17.38 $\pm$ 1.655
750	22.75 $\pm$ 0.425	21.01 $\pm$ 0.850
1000	41.87 $\pm$ 2.790	45.95 $\pm$ 1.874
1500	63.89 $\pm$ 2.730	70.73 $\pm$ 3.776

Values are expressed as Mean  $\pm$  SEM, n=3. Similar activity pattern between *L. cyanescens* extract and catechin was at  $p > 0.05$

mixture was mixed with  $\text{FeCl}_3$  (0.1%, 0.5 ml) and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated reductive potential of the extract.

#### Total phenolic content (TPC)

Total phenolic content was determined using the Folin-ciocalteu assay, following the method of Singleton and Rossi (1965). In total, 10 to 1000  $\mu\text{g}$  of the extract (1 mg/ml) was added to 1 ml of Folin ciocalteu reagent. It was allowed to stand for 3 min after mixing. Saturated  $\text{Na}_2\text{CO}_3$  solution (11 ml) was added and the solution was made up to 10 ml with distilled water. The final mixture was mixed and then incubated for 90 min in the dark at room temperature. A reagent blank was prepared using distilled water. The absorbance was measured spectrophotometrically at 725 nm. Total phenolic values are expressed in terms of catechin equivalents (CE) in milligrams per gram plant extract.

All determinations were made in triplicate. Correlation plots of total phenolic content against the antioxidant activities by DPPH and FRAP models were obtained.

#### Total flavonoid content (TFC)

The total flavonoid content in extracts was determined according to Jia et al. (1999), briefly, to various concentrations of distilled water and sample,  $\text{NaNO}_2$  (75  $\mu\text{l}$ , 5%) was added and left to stand for 5 min. Hydrated aluminium chloride (10%, 150  $\mu\text{l}$ ) was added and left to stand for another 6 min. NaOH (500  $\mu\text{l}$ , 1 M) and 275  $\mu\text{l}$  of distilled water were added and mixed. The colour intensity was read spectrophotometrically at 500 nm. All determinations were performed in triplicate.

Total flavonoid values are expressed in terms of catechin equivalents (CE) per gram of plant extract from a standard curve. Correlations plots of total flavonoid content against its antioxidant activities by DPPH and FRAP models were obtained.

#### Column chromatographic separation of extracts

Dried acetone extract (3 g) was separated into different fractions by column chromatographic technique with silica gel as stationary phase. A wet column packing was done and the separation was carried out with hexane and ethyl acetate in the gradient of 90:10 (hexane: ethyl acetate) to 100% ethyl acetate and finally washed with methanol.

The pattern of the separation was monitored by thin layer chromatographic fingerprint and similar patterns were pooled together to obtain six fractions (F1, F2, F3, F4, F5 and F6).  $^1\text{H}$  and infrared spectral data were obtained for the most active fraction (F5) to determine the nature of compound it contained.

#### Bioassay of fractions

Antioxidant properties of the different fractions were determined by measuring the free radical scavenging activity using the stable radical (DPPH) according to the method described above. The comparative antioxidant activities of the fractions were obtained from a statistical plot of their activities at different concentrations.

#### Statistical analysis

The results are given as mean  $\pm$  standard error of mean (SEM). The experiments were done in triplicate. Student t-test was used for comparison of two mean values and one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test was applied in the analysis of data with more than 2 mean values.  $P < 0.05$  was taken as significant using GraphPad Prism 5 software.

## RESULTS

350 g of dried *L. cyanescens* extract yielded 26 g of dried extract (7.4%). The mobile system of hexane: ethyl acetate (5:5) (H: E) was most appropriate for the study.

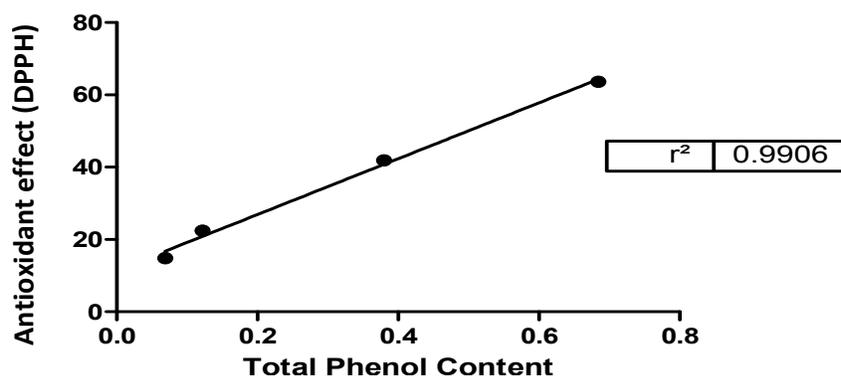
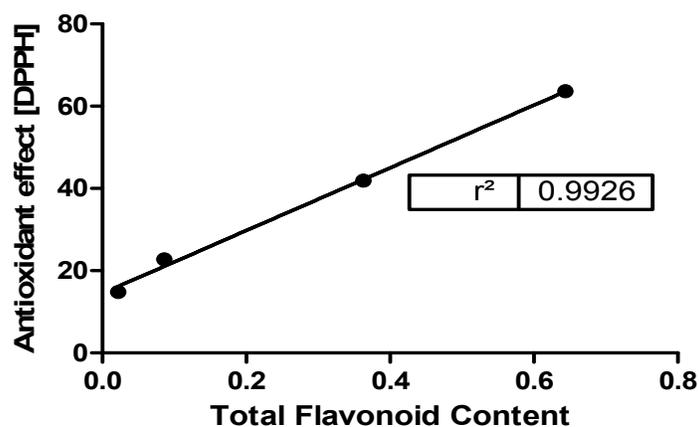
#### Antioxidant assay

As shown in Tables 1 and 2, the antioxidant activity of the extract was concentration-dependent. Scavenging effects of *L. cyanescens* on DPPH radical increased with increase in concentration. At 0.50 mg/ml, scavenging effect of *L. cyanescens* was 14.83% while catechin was 17.38%. At 1.5 mg/ml, scavenging effect of *L. cyanescens* was 63.89% and that of catechin was 70.73%. A similar pattern was obtained in the FRAP method as shown in Table 2. *L. cyanescens* extract

**Table 2.** Ferric reducing-antioxidant power (FRAP) capacity of *L. cyanescens* extract.

Concentration ( $\mu\text{g/ml}$ )	<i>L. cyanescens</i>	Catechin	T- test (P value)
100	$0.069 \pm 0.003$	$0.071 \pm 0.008$	0.188
500	$0.221 \pm 0.002$	$0.314 \pm 0.016$	0.878
750	$0.340 \pm 0.020$	$0.483 \pm 0.036$	0.032
1000	$0.435 \pm 0.008$	$0.610 \pm 0.011$	0.008

Values are expressed as Mean  $\pm$  SEM, n=3 in each group. Similar activity pattern between *L. cyanescens* extract and catechin was at  $p > 0.05$ .

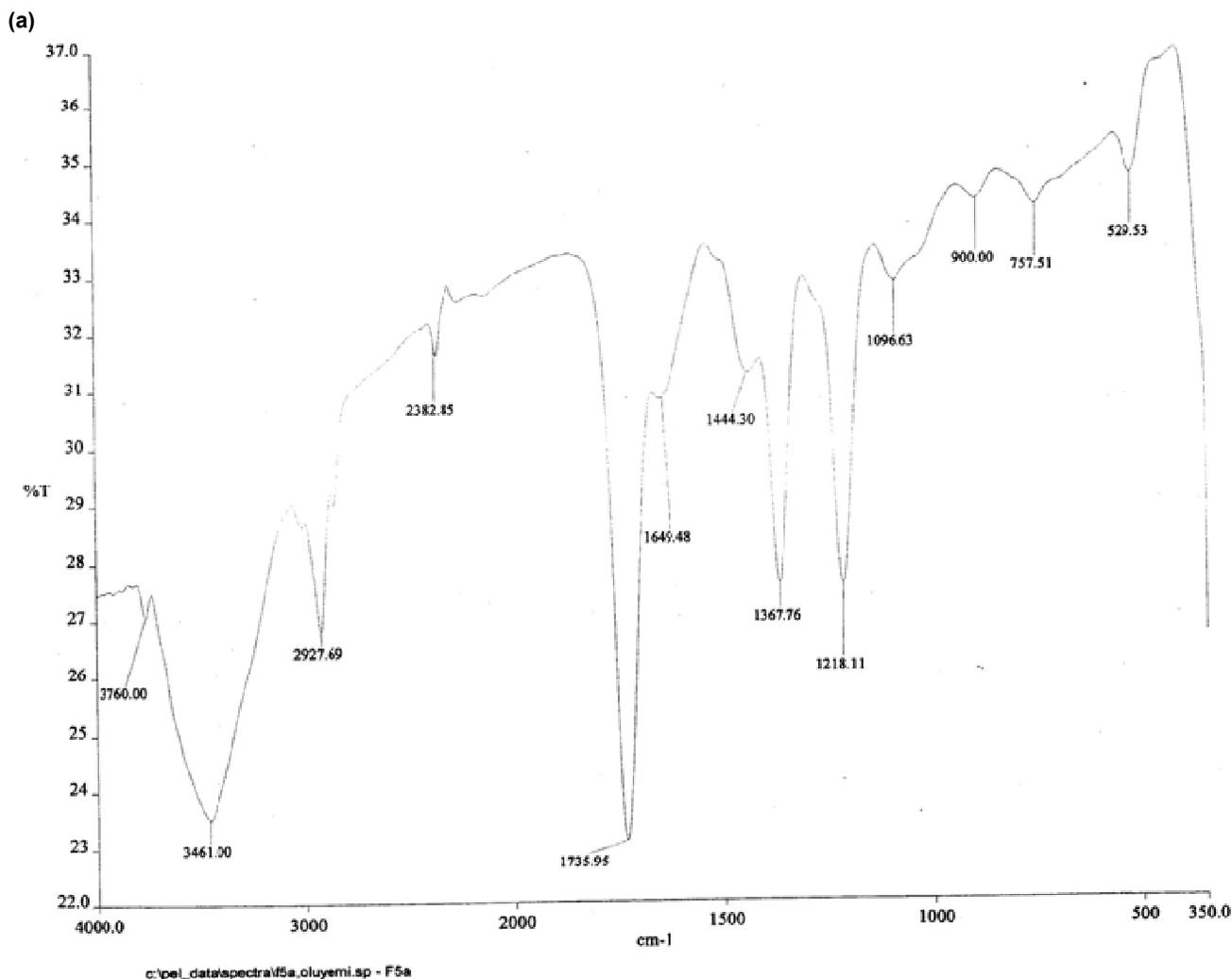
**Figure 1.** Correlation between DPPH and TPC of *Lonchocarpus cyanescens* benth leaves extracts.**Figure 2.** Correlation between DPPH and TFC of *Lonchocarpus cyanescens* benth leaves extracts.

showed comparative antioxidant activity to catechin in DPPH and FRAP methods  $P > 0.05$ .

#### Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC of *L. cyanescens* obtained were correlated

with the antioxidant activities (DPPH and FRAP). The correlations for TPC and TFC with DPPH are shown in Figures 1 and 2. We observed a strong correlation between antioxidant activity (DPPH method) of the extract and phenol and the flavonoid contents (Figures 1 and 2) with a correlation coefficient of 0.9906 and 0.9926, respectively. While the same pattern was observed with



**Figure 3a.** IR spectra of the most active fraction of *L. cyanescens* [F5].

the FRAP method, there was a lower correlation to the total phenol and flavonoid content observed (0.8635 and 0.8840, respectively) than in the DPPH method.

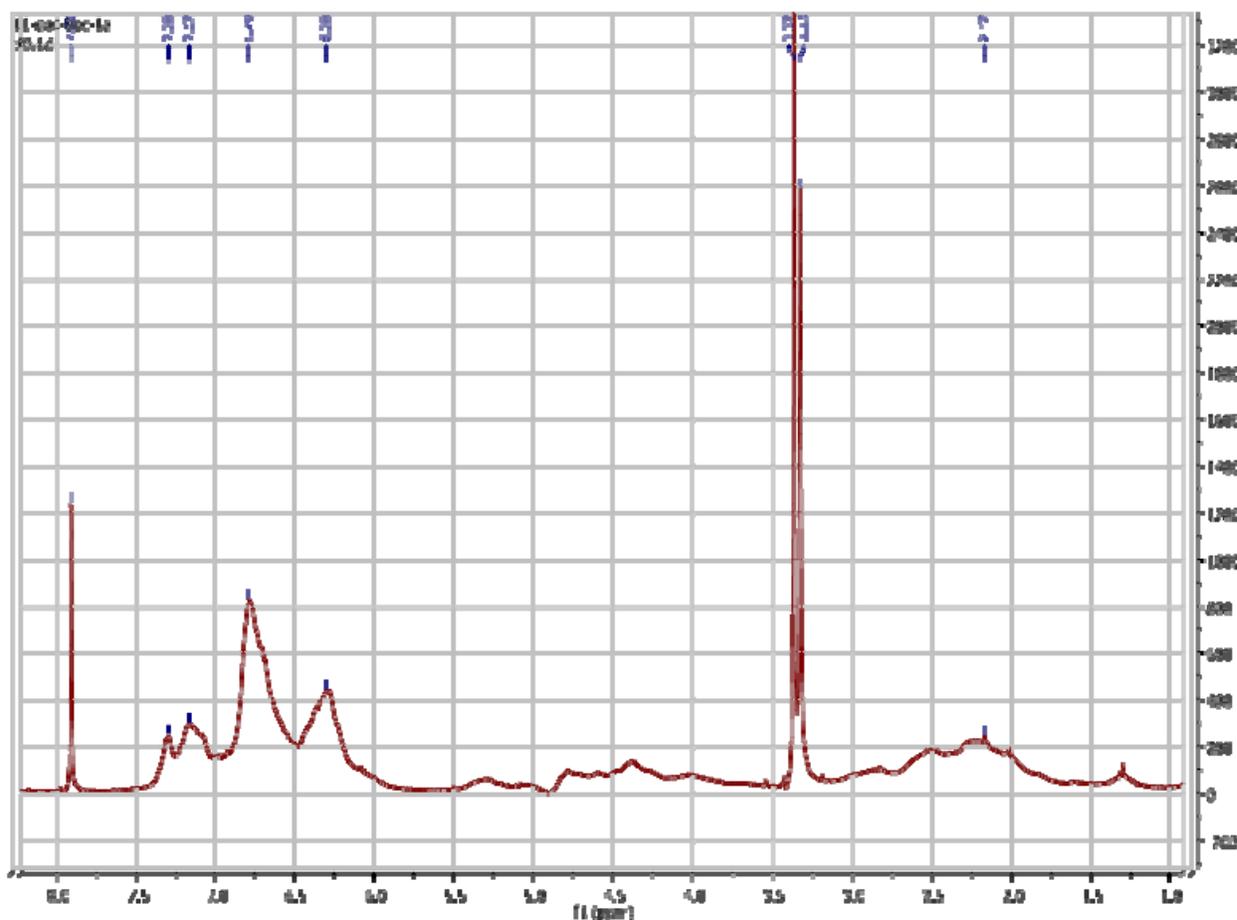
#### Activity of chromatographic fractions

As shown in Figure 4, the most active fraction was F5. There is a significant difference between the activity of F5 and the other inactive fractions such as F2 and F3 ( $p < 0.05$ ). Fraction F6 also showed significant activity.  $^1\text{H}$  NMR spectra showed downfield signals between 6 and 8 ppm, while Infrared spectra indicated the following functional groups - OH, C=O and C-O as shown in Figure 3. These signal patterns are associated with flavonoids.

#### DISCUSSION

DPPH free radical method is based on the determination of the concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at steady state in solution. After the addition of the antioxidant, the concentration of DPPH which absorbs at 517 nm is reduced by the antioxidant; while in the ferric reducing antioxidant power (FRAP) assay, the antioxidant principle is based on the ability of the antioxidant to reduce ferric (III) ions to ferrous (II) ions. The two methods are fast and sensitive methods that are readily applicable in the study of plant extracts for their antioxidant activities in the search for bioactive compounds against diseases associated with oxidative stress (Azizah et al., 2010). The antioxidant activities of

(b)

<sup>1</sup>H NMR Spectrum

**Figure 3b.** <sup>1</sup>HMR spectra of the most active fraction of *L. cyanescens* [F5].

the extract which is comparable to catechin in both studies highlighted its potential. This is because catechin related compounds have a central place in antioxidant and cancer related research (Menon et al., 1999).

Plant polyphenols which constitute a diverse group of compounds (flavonols, flavanol, stilbenes, phenolic acids, e.t.c) possess an ideal structure for free radical scavenging activity. This family of molecules has a remarkable pharmacological importance as therapeutic agents. They have been used directly or through some of their derivatives in various disease conditions including cancer, arthritic condition, among others (Gomes et al., 2012; Izzi et al., 2012). The fact that they have antioxidant activities by acting through several ways is central in the explanation of their diverse pharmacological actions (Villaño et al., 2007). Very good correlation observed between the phenolic/flavonoid contents and

the antioxidant activity confirmed that the observed activity in *L. cyanescens* extract was majorly due to the flavonoid content. The most active fraction as indicated from preliminary spectroscopic investigations (IR and <sup>1</sup>NMR) as shown in Figure 3 contained essentially phyto-phenolic compounds (flavonoids). Our findings possibly account for the use of this plant in the treatment arthritic conditions and ulcers (Ismot et al., 2013; Iwu and Ayanwu, 1982).

While there is no report on any characterized flavonoid from this specie [*L. cyanescens*], studies on other species of *Lonchocarpus* genus have led to the isolation of some phyto-phenolic compounds. Jean-Robert et al. (2001) previously isolated some phenolic compounds from *Lonchocarpus chiricanus* which include chiricanine. Cassidy and Setzer (2010) investigated the anti-cancer potential of some flavonoids from *Lonchocarpus haberi*.

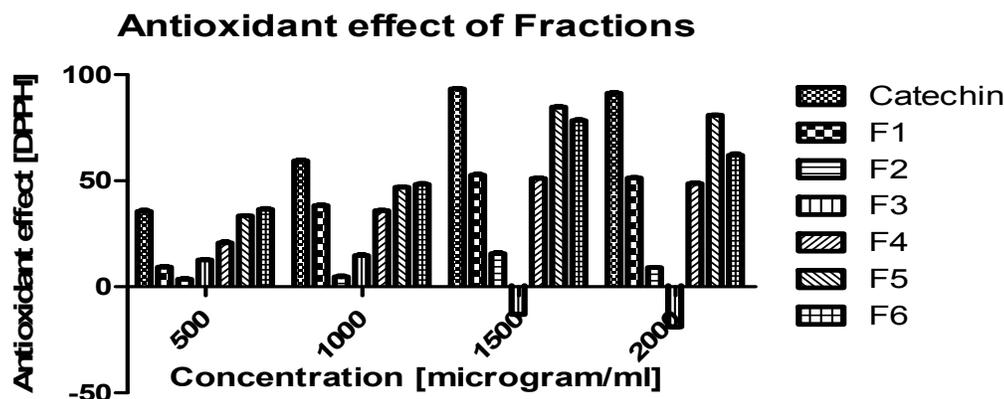


Figure 4. Comparative antioxidant activities of chromatographic fractions by DPPH method.

Such related compounds isolated from the other species in this genus are possibly associated with the antioxidant activity we have observed in the extract of *L. cyanescens*.

In the correlation studies, a better correlation was observed with the DPPH than the FRAP model. The mode of action of the two methods are closely related but several authors have found their sensitivity slightly different, although the difference in the two methods is not statistically significant which is similar to what we observed in this study (Christina et al., 2011).

The fact that more than one fraction (fractions 5 and 6) showed good activity suggests that the mode of action is possibly synergistic. There are possibly several phyto-phenolic compounds in this extract, since our correlation studies showed the antioxidant activities are essentially through this class of compounds. Previous studies on the bioactivity of *Ochna pretoriensis* indicated a similar synergistic pattern (Makhafola et al., 2012). Comparative antioxidant activity of the whole extract and the most active fraction of *L. cyanescens* were 14.83, 41.87, 63.89 and 33.37, 46.79, 84.80 respectively at 500, 1000 and 1500 µg/ml. Some extracts are better applied as whole, but that does not diminish the importance of isolating bioactive compounds from whole extracts because of the potential of obtaining lead compounds for the design and synthesis of drug candidates with increased activity and reduced toxicity.

## Conclusion

This study shows that *L. cyanescens* extract have significant antioxidant and free radical scavenging activities. Antioxidant-rich plant extracts serves as sources of nutraceuticals that alleviate oxidative stress and therefore prevent or slow down degenerative diseases. This possibly explains the basis for the local

application of this plant in patho-physiological conditions associated with oxidative stress which include ulcer, arthritis, neurodegenerative disorders, among others. <sup>1</sup>H and Infra-red spectra indicated the presence of flavonoid in the most active fraction. Further studies are required on the *in vivo* antioxidant activity and toxicity profile of these extract and fractions before its consideration as a phyto-drug.

## Conflict of Interests

The author(s) have not declared any conflict of interests

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