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Bioguided isolation of an antioxidant compound from *Combretum racemosum* P.Beav leaf

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ABSTRACT

Free radicals are known to cause damage to the body cells leading to many diseases. These cells can be protected from such deleterious effects by antioxidants; therefore, plant derived antioxidants are of global interest. *Combretum racemosum* have been used traditionally in the treatment of ulcers, cancers and infections. Current studies investigated the antioxidant activities of this plant extract and its fractions. The antioxidant activity of the crude extract was determined by Ferric Reducing Power Assay, DPPH free radical scavenging assay, Total Phenolic and Total Flavonoid Content methods. Bioguided column chromatographic separation was carried using DPPH free radical scavenging assay to assess activity of fractions. A bioactive compound was isolated and characterized by application of Spectroscopic techniques (ESI-MS, HRMS, 1D and 2D NMR). Bioactivity of the crude extract showed that *Combretum racemosum* has good antioxidant activity which is comparable to the reference antioxidant (catechin). Bioactivity guided fractionation using DPPH method led to the isolation of 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol as an active principle from the extract. This study suggests that there is a scientific basis for the application of *C. racemosum* extract in the management of diseases that are associated with cell damage caused by free radicals.

Keywords: *Combretum racemosum*, DPPH, FRAP, Antioxidant, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol.

INTRODUCTION

Species of *Combretum* genus has been of interest in the last two decades because of the isolation of some compounds with highly significant activities in anticancer and anti-infectives models isolated from this genus (Okwuosa et al., 2012; Pettit et al., 1997). This has made the *combretum* genus a very important one in search of bioactive compounds for the treatment and management of disease conditions that are related to Reactive Oxygen Species (ROS). Oxygen, though not deleterious in itself, is involved in the generation of various kinds of ROS. ROS formed during metabolism or through the action of ionizing radiation can interact with bio-molecules and ultimately lead to an onset of degenerative diseases such as cancers,

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inflammation, premature aging, ulcers, cardiovascular diseases and other illnesses (Aliyu et al., 2009; Afanaslev et al., 1989).

Some species of the *Combretum* genus in Africa have been used over the years for diseases in these categories (ulcers and cancers) but very little is known of the relationship between their activity and the secondary metabolites they contain. *Combretum racemosum* is one of such species. It is used locally for the treatment of ulcers, infections, among other diseases. There are also recent reports of the anticancer potential of this species (Okwuosa et al., 2012).

Current study investigated the antioxidant potential of *Combretum racemosum* extract and its fractions which led to the isolation of a compound from the most active fraction.

MATERIALS AND METHODS

General methods

Nuclear Magnetic Resonance (NMR) data were obtained on a Bruker 500 MHZ model using Deuterated methanol as solvent and TMS as internal standard. Electrospray Ionization Mass Spec (ESI-MS) and High Resolution Mass Spec (HRMS) data were obtained from Agilent Mass Spectrophotometer. (All Spectral data were obtained from the Facilities of Indian Institute of Integrative Medicine, Jammu, India).

Plant material

Fresh leaves of *Combretum racemosum* was collected from the Botanical Garden of the University of Ibadan. The Curator of the Botanical Garden identified the sample and Voucher specimen was deposited at the Herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan. The leaves were air dried, milled into fine powders and stored in closed glass bottles in the dark cupboard until use.

Extraction of *Combretum racemosum* plant material

The dried milled leaf (440 g) of *C. racemosum* was extracted with 4.4 litres of acetone by cold maceration for 72 hours with intermittent shaking. The extract was filtered and concentrated with a Rotary evaporator to minimum volume at 40 ^{0}C. The extract was transferred to a pre-weighed glass container and dried completely with a vacuum oven at 40 °C.

Antioxidant assays

Ferric Reducing Power (FRAP) assay

This was determined according to the method described by 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 ^{0}C for 20 min. Trichloroacetic acid (10%, 2.5 ml) was added to the mixture. A portion of the resulting mixture was mixed with $FeCl_3$ (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.

DPPH radical 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 (1985). Various concentrations of the extracts were added to 4 ml of methanol solution of DPPH (1 mM, 1ml). 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:

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

A control= Absorbance of control
A test= Absorbance of test sample

Total phenolic content (TPC)

Total Phenolics was determined using the Folin-ciocalteu assay (Singleton and Rossi, 1965). In total, 10 µg to 1000 µg 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 Na_2CO_3 solution (1.1 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 mins 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.

Total flavonoid content (TFC)

The total Flavonoid content in extracts was determined according to Jia et al. (1999). To various concentrations of sample and distilled water, $NaNO_2$ (75 µl, 5%) was added and left to stand for 5 mins. Hydrated alluminium chloride (10%, 150 µl) was added and left to stand for another 6 min. NaOH (500 µl, 1M) and 275 µl 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. A standard curve of catechin was plotted.

Column chromatographic separations and screening of fractions

Acetone extract (3 g) was eluted on 59 g of silica gel 60-120 using a gradient elution procedure with 100% hexane and an increasing concentration of ethyl acetate 10%, 20%, 30%, 40%, 50%, 60, 70, 80, 90%, 100%. A total of 76 fractions were collected. The pattern of the separation was monitored by Thin Layer Chromatography (TLC) fingerprint and similar patterns were pooled together to obtain 7 fractions (F1 – F7).

Bioactivity of the fractions was carried out by DPPH model. The most active fraction which contained essentially a compound was washed in a mixture of methanol and chloroform to obtain a yellowish amorphous compound. Spectral data (IR, ESI-MS, HRMS, 1D and 2D NMR) were obtained for the compound and the structure of the compound **(1)** elucidated.

Statistical analysis

The experiments were done in triplicate. The results are given as mean ±standard deviation (SD). Student's t-test was used for comparison between two means and One- way Analysis of Variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant at $p < 0.05$ (GraphPad prism 5 was used).

RESULTS

Reducing power assay (FRAP)

The reducing capacity of an extract is an indicator of its antioxidant potential. A higher absorbance value is an indicator of increased reducing power. The higher absorbance value observed at high concentrations indicates a strong reducing power potential of the extract. A dose dependent increase in absorption shown in Table 1 further highlights the antioxidant potential of this extract. The extract showed a higher reducing power than was observed in the reference compound. For example, at the concentration of 1000 µg/ml, the extract had an absorbance value of 2.111±0.120 while the

reference compound had a value of 0.610±0.019.

DPPH scavenging activity

As shown in Table 2, the acetone extract of *C. racemosum* showed a comparable activity to the reference standard, which is an indicator of the potential of this extract as a natural source of antioxidant.

Determination of total flavonoid content (TFC)

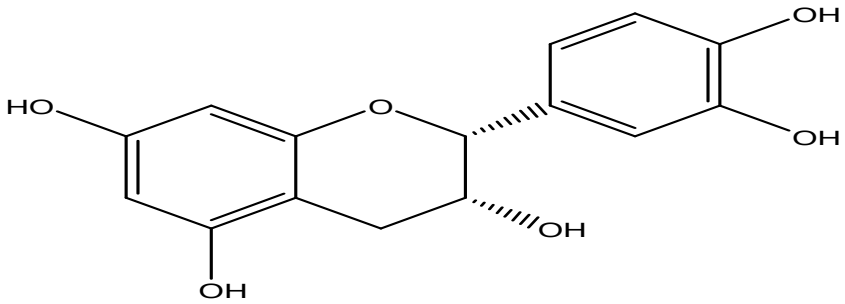
The total Flavonoid content of *C. racemosum* as determined from the catechin calibration curve (Y=0.0002x + 0.0096, r^2 = 0.9908) was 7815.33 mg Catechin/ml. A correlation plot of TFC and antioxidant activities (FRAP) is shown in Figure 1 with a correlation coefficient of 0.9947, While a Correlation plot of TFC and the DPPH model of antioxidant activity is shown in Figure 2 with a correlation coefficient of 0.9140.

Total phenol content

The Total Phenol Content of *Combretum racemosum* as determined from the catechin calibration curve Y = 0.0007x + 0.439, r^2 = 0.974 was 2997mg Catechin/ml. A correlation plot of TPC and antioxidant activities (FRAP) had a correlation coefficient of 0.9222, while a Correlation plot of TPC and the DPPH model of antioxidant activity had a correlation coefficient of 0.8103.

Antioxidant activities of fractions

Figure 3 showed that Fraction 7 had the highest activity. The activity of fraction 7 is significantly higher than the other inactive fractions. Four fractions are inactive (F1 – F4) while Fraction 6 showed significant activity when compared to the other inactive fractions ($p < 0.05$).



(1)

Table 1: Reducing power (FRAP) of acetone extract of *C. racemosum* and Catechin.

Concentration (µg/ml)	CRAE	Catechin	T-TEST (P value)
50		0.153±0.027	0.056±0.008
0.0403			
100		0.243±0.039	0.071±0.013
0.0237			
500		1.218±0.023	0.314±0.029
0.0002			
1000		2.111±0.120	0.610±0.019
0.0019			

CRAE – *Combretum racemosum* acetone extract, n=3

Table 2: % inhibition of DPPH radical by *C. racemosum* extract and catechin.

Concentration (µg/ml)	CRAE	Catechin
100	25.88±0.077	35.58±0.027
500	32.22±0.028	40.59±0.015
1000	55.72±0.061	53.39±0.020
2000	60.15±0.073	69.70±0.009

CRAE = Combretum racemosum acetone extract, n=3, p>0.05.

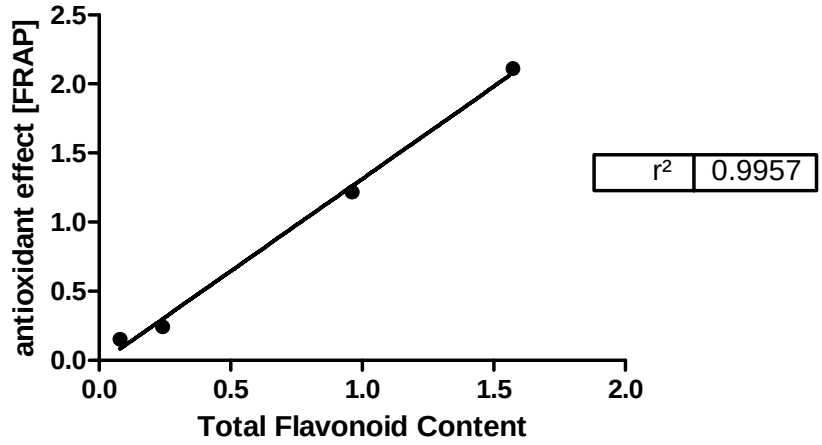


Figure 1: Correlation between FRAP and Total Flavonoid Content. The r^2 values of 0.9957 indicated that the flavonoid content is the major determinant of the antioxidant activity.

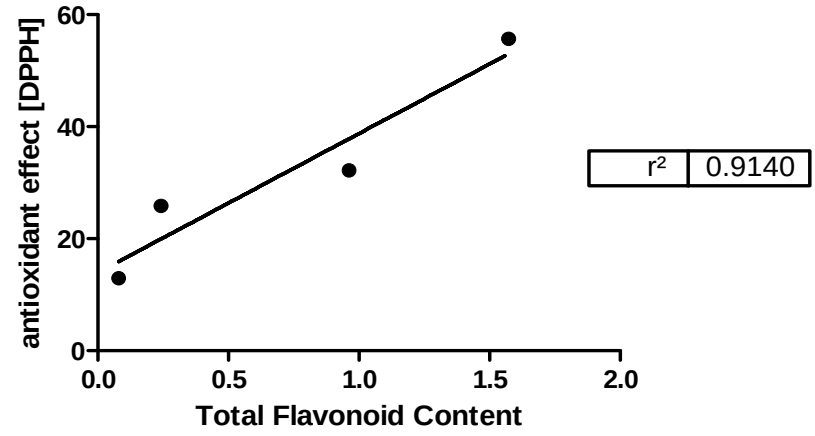


Figure 2: Correlation between DPPH and TFC. A good correlation was shown between the Total Flavonoid Content and antioxidant activity.

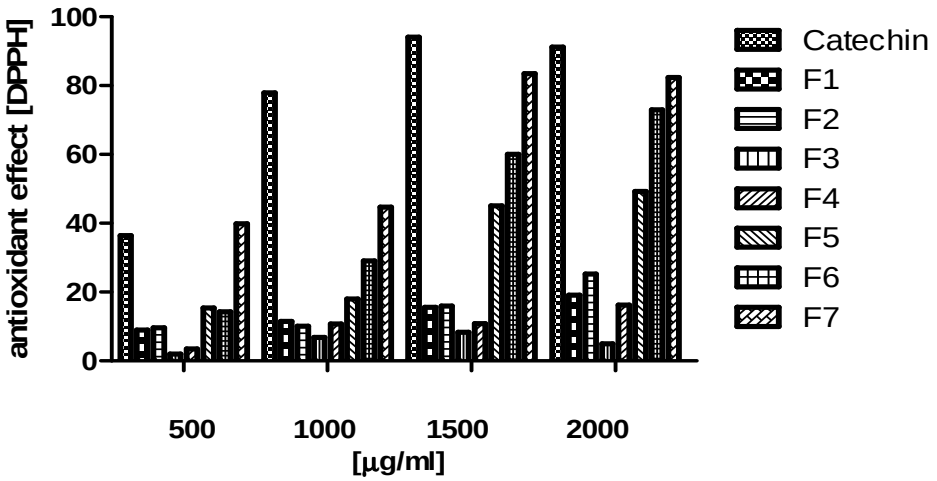


Figure 3: Antioxidant activity of different fractions of *C. racemosum* (DPPH). Fraction F7 showed the highest activity while F1 –F4 were inactive.

DISCUSSION

DPPH and FRAP assay procedures are reputed as quick, reliable and reproducible parameters to investigate the *in vitro* antioxidant properties of plant extracts and food products (Pellegrini et al., 1999). The observed activities of *Combretum racemosum* extract and fractions through these methods have shown its antioxidant potential (Odabasoglu et al., 2004). Flavonoids are antioxidants that neutralize a variety of free radicals including nitric oxide, hydroxyl radical (HORAC), while phenolic constituents can react with active oxygen radicals such as hydroxyl radical, superoxide anion radical and lipid peroxy radical (Koleva et al., 2002). The correlation of the Total Flavonoid Content with the antioxidant activities (0.9957 and 0.9140 in FRAP and DPPH methods respectively) clearly demonstrated that the antioxidant effect of the extract is largely due to its flavonoid content. The correlation of antioxidant activities with the Total Phenol Content is lower than with the Flavonoid Content (0.9222 and 0.8103 in FRAP and DPPH methods respectively). This is possibly because the Total Phenol Content assay is broader in sensitivity to other functional groups than the Flavonoid assay which is more specific for the flavonoids. Within the limit of our observation, we can reasonably infer that the activity of the extract is due to some flavonoids present in it.

It's already an established fact that Gastric mucosa damage by *Helicobacter pylori* is mediated through Reactive Oxygen Species, either through excessive synthesis of ROS or deficiency of antioxidant defences (Davies et al., 1994). The antioxidant effect we have observed could possibly account for the antiulcer activity previously reported for *Combretum racemosum* extract (Okwuosa et al., 2006).

Structural elucidation of isolated compound

ESI- MS indicated the isolated compound have *m/z* 291 $(M+H)^+$ corresponding to m/z 290, while HRMS m/z 291.0861 $(M+H)^+$ (calculated 291.0863 for $C_{15}H_{15}O_6^+$) confirmed the molecular formulae to be $C_{15}H_{14}O_6$. The ^1H and ^{13}C Spectra data for the compound clearly indicated a monoflavonoid with prominent and

characteristic downfield signals. Correlation of the obtained data with previously isolated flavonoids confirmed the isolated compound **(1)** to be 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol isolated from *Carapa guianensis* (Shu-Hua et al., 2003). 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (commonly known as epicatechin) is associated with very significant activities in biological systems (Weyant et al., 2001). The underlying principle is most probably associated with its antioxidant activities discussed in this work.

2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol has been isolated in this study as one of the compounds with antioxidant activities in *C. racemosum* extract, while effort is ongoing to isolate and purify other compounds with related activities from this extract.

Ongoing studies by our group indicated the antibacterial potential of *Combretum racemosum* extract. These studies further highlight the need for further investigation of unexplored *Combretum* species of Africa and particularly *Combretum racemosum*.

Conclusion

The combretaceae family is significant in the search for bioactive compounds for disease conditions that are associated with oxidative stress. This study showed the antioxidant potential of *Combretum racemosum* through the DPPH and FRAP methods. The clear correlation between the Total Flavonoid Content and antioxidant activities of the extract strongly suggests that the activity is largely due to the flavonoid content.

A flavonoid, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol was isolated as a major antioxidant principle from *Combretum racemosum* extract. It is postulated that the antioxidant activity of this extract is a consequence of synergistic effect of some phyto-phenolic compounds present in the extract. Previous studies by our group indicated such synergistic pattern in other species (Makhafola et al., 2012; Samuel et al., 2009). *In vivo* studies and detailed toxicological studies need to be carried out on the extract/fractions before consideration for development as a Phyto-drug.

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