CHAPTER FOUR
ANTIOXIDANT CAPACITY OF DIETARY PLANTS OF THE BLACK RHINOCEROS

4.1 Introduction

The antioxidant activity of phenolic compounds is mainly due to the redox properties that allow them to act as reducing agents, oxidizing agents, hydrogen donators and metal chelators (Villaño *et al.*, 2005; Tawaha *et al.*, 2007). A compound can transfer a hydrogen atom or single electron to reduce an oxidant. The antioxidant activity of phenolic compounds depends mainly on the structural configuration of the molecule, such as the number and position of the available hydroxyl groups (Paixão *et al.*, 2007).

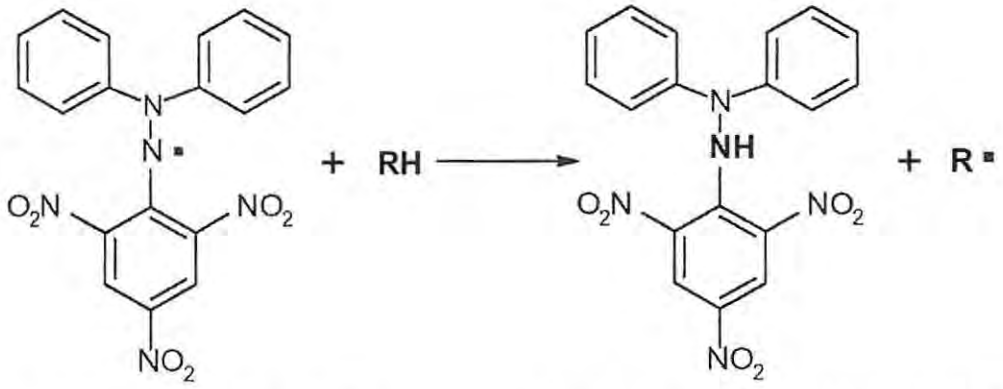
Many phenolic compounds have been assayed for their antioxidant capacity using different analytical methods such as 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid) ($ABTS^{\cdot+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and the ferric reducing antioxidant potentials (FRAP) (Villaño *et al.*, 2005; Paixão *et al.*, 2007). These assays use different chemical reactions, principles and experimental conditions, which may lead to variable results when attempting to compare assays (Paixão *et al.*, 2007).

Certain limitations such as slow reaction rates (Brand-Williams *et al.*, 1995), solubility problems of certain antioxidants and possible interference from organic acids may be encountered when these assays are used (Fukumoto and Mazza, 2000). Furthermore, absolute values of antioxidant activities may vary from one study to another, causing difficulty when comparing single compounds even when the same method is used (Re *et al.*, 1999; Paixão *et al.*, 2007). Another difficulty is that the antioxidant activities of pure phenolic compounds are expressed in different terms and are therefore difficult to compare (Villaño *et al.*, 2005; Paixão *et al.*, 2007). The main limitation in using antioxidant activity assays is that they may not be representative of antioxidant activities *in vivo* (Villaño *et al.*, 2007; Choi *et al.*, 2007). However, antioxidant activity assays are useful as indicators of the antioxidant capacity of dietary components prior to consumption (Villaño *et al.*, 2007).

Certain assays utilize free radical chromogens, which are quenched in the presence of antioxidants and may lead to a decrease in absorbance. Widely used chromogens are $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, both of which show excellent stability under certain assay conditions and also show important differences in response to different antioxidants (Arnao, 2000; Samarth *et al.*, 2008). Alternatively, the Folin-Ciocalteu assay has been used to measure the total phenolics in various natural products and beverages (Davalos *et al.*, 2003; Katalinic *et al.*, 2006; Tawaha *et al.*, 2007; Paixão *et al.*, 2007; Villaño *et al.*, 2007). The FRAP assay directly measures the redox potential of an antioxidant (Halvorsen *et al.*, 2002).

4.2 The $DPPH^{\cdot}$ method

DPPH is a violet-coloured stable free radical that was discovered by Goldschmidt and Renn (1922) (cited by Ionita, 2005) and is used as a colorimetric reagent for redox reactions. This reagent does not react with oxygen and can be kept indefinitely with little decomposition. It has been used in a variety of investigations such as the determination of antioxidant properties of amines, phenols and natural compounds such as vitamins, plant extracts and medicinal drugs. When the DPPH radical is reduced, its decolourisation can be measured at room temperature and at a wavelength of 515 nm (Brand-Williams *et al.*, 1995; Arnao, 2000; Argolo *et al.*, 2004; Chen *et al.*, 2006; Paixão *et al.*, 2007). The reduction of the $DPPH^{\cdot}$ depends on the ability of a substance or a complex mixture of substances to donate either hydrogen atoms or electrons to the radical. Reduction of $DPPH^{\cdot}$ may lead to the formation of a free radical ($R^{\cdot}$) and a reduced form of $DPPH^{\cdot}$ as shown in Fig. 4.1. The free radical produced can undergo further reactions if it is not completely eliminated. The decolourization of the $DPPH^{\cdot}$ is an indication of the number of DPPH molecules reduced by the antioxidant molecule (Arnao, 2000; Paixão *et al.*, 2007).



diphenylpycrylhydrazyl (radical) diphenylpycrylhydrazine (nonradical)

Figure 4.1: Structure of a DPPH radical undergoing reduction by an antioxidant molecule (adapted from Paixão *et al.*, 2007).

The DPPH radical method is rapid and simple, when the DPPH$^{\cdot}$ is dissolved in an organic solvent, which does not interfere with the DPPH assay. Interference could lead to underestimation of the effectiveness of certain antioxidant compounds in the reaction mixture (Brand-Williams *et al.*, 1995; Arnao, 2000; Paixão *et al.*, 2007).

The mechanism by which antioxidants react with the DPPH$^{\cdot}$ may differ and the scavenging activity of different compounds depends on the structure of the antioxidant (Brand-Williams *et al.*, 1995). Furthermore, certain antioxidants react quickly while others react more slowly with DPPH$^{\cdot}$ due to its stability. As a result, kinetic assays may be useful for slow reacting compounds although the reaction rate may not be linear (Brand-Williams *et al.*, 1995; Bondet *et al.*, 1997; Paixão *et al.*, 2007).

4.3 The $ABTS^{\cdot+}$ method

The ABTS assay is rapid and requires limited technical experience to enable the processing of a large number of samples (Paixão *et al.*, 2007). $ABTS^{\cdot+}$ is a stable cation chromogen free radical, which can be generated using either manganese dioxide or potassium persulfate (Miller and Rice-Evans, 1997a; Denis *et al.*, 2004). This radical is soluble in both aqueous and organic solutions, thus, both hydrophobic and hydrophilic compounds can be measured readily (Arnao, 2000; Re *et al.*, 1999; Nenaids *et al.*, 2004). This radical can be employed against a wide range of

compounds such as plant extracts, beverages and biological fluids (Re *et al.*, 1999; Nenaids *et al.*, 2004; Villaño *et al.*, 2005; Katalinic *et al.*, 2006).

The ABTS radical assay is based on the ability of an antioxidant to donate a hydrogen atom to the radical cation. The reduction of blue-green $ABTS^{\cdot+}$ cation is measured by a decrease in absorption at a wavelength of 734 nm (Miller and Rice-Evans, 1997a & b; Re *et al.*, 1999; Nenaids *et al.*, 2004). Different researchers have modified the assay by dissolving the $ABTS^{\cdot+}$ in inorganic solvents or phosphate buffered saline (PBS). This may be the reason for the lack of consistency of the results between various laboratories (Nenaids *et al.*, 2004; Villaño *et al.*, 2005). Furthermore, an antioxidant reduces $ABTS^{\cdot+}$ in a manner dependent on the time of the reaction, on the antioxidant activity of the sample and concentration of antioxidant (Miller and Rice-Evans, 1997a & b; Re *et al.*, 1999).

4.4 The FRAP assay

This assay directly measures reductants in a sample (Halvorsen *et al.*, 2002). The method was initially used to measure plasma antioxidants (Benzie and Strain, 1996) and was further used to assay pure compounds (Pulido *et al.*, 2000). Many phenolic compounds, including plant extracts can be assayed for their ability to reduce ferric ion-2,4,6-tri-2-pyridyl-s-triazine (Fe^{3+}-TPTZ) complex to ferrous ion-2,4,6-tri-2-pyridyl-s-triazine (Fe^{2+}-TPTZ) complex. This method takes advantage of the oxidation-reduction abilities of reductants and is applicable to both aqueous and alcohol extracts of different plants (Pulido *et al.*, 2000; Wong *et al.*, 2006). However, a possible limitation of this method is that it does not react with thiols found in plants, which emphasizes the lack of accuracy for certain crude samples (Halvorsen *et al.*, 2002).

4.5 The Folin-Ciocalteu assay

This assay is rapid, reproducible and can be used to assess the phenolic content of a wide range of samples at the same time (Paixão *et al.*, 2007; Tawaha *et al*, 2007). In principle, the assay measures the ability of various phenolic compounds to reduce a

phosphotungstate-phosphomolybdate complex, which results in the formation of blue coloured reaction products that can be measured at 765 nm (Paixão *et al.*, 2007). The reduction of the phosphotungstate-phosphomolybdate complex depends on the number of phenolic hydroxyl groups available (Singleton and Rossie, 1965; Katalinic *et al.*, 2006; Tawaha *et al.*, 2007).

Not all the phenolic compounds in an extract may contribute equally to the overall reduction of the Folin reagent as certain extracts with a high number of phenolic compounds may give either low or high values (Tawaha *et al.*, 2007). A limitation of this assay is that it is not specific when estimating the total phenolic content in a mixed sample (Paixão *et al.*, 2007). Furthermore, interference from sugars, lipids and chlorophyll may overexpress the total phenolic compounds obtained in a given sample under certain circumstances (Luximon-Ramma *et al.*, 2002; Dávalos *et al.*, 2003).

In the present investigation, plants found to be part of the diet of the black rhinoceros by molecular analysis of dung and by observation studies, were assessed for their antioxidant capacity using DPPH, ABTS and FRAP assays. The Folin-Ciocalteu method was used to measure the total phenolic content of these plants. This study attempted to ascertain a possible relationship between black rhinoceros browse and the antioxidant capacity of this browse.

4.6 Materials and methods

4.6.1 Reagents

Methanol, hydrochloric acid, acetic acid, sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate and sodium carbonate (anhydrous) were purchased from Merck (South Africa). 2,2-diphenyl-1-picryl-hydrazyl, gallic acid, Folin–Ciocalteu's phenol reagent, 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid), potassium persulfate, ferric chloride, 2,4,6-trypyridil-*s*-triazine and ferrous sulfate heptahydrate were purchased from Sigma Aldrich (Germany).

4.6.2 Preparation of plant extracts for antioxidant activity assays

Leaves of twenty-five plant species reported to form part of the diet of the black rhinoceros were collected from the GFRR. These plants were assessed for their free radical scavenging activities, ferric reducing abilities and for total phenolic content. The plants were identified and classified at the Selmer Schonland Herbarium in Grahamstown.

The leaves of the plants collected were placed in separate Ziploc plastic bags (10 x 25 cm) with silica gel distributed between the layers of the leaves and each sample was stored at –20°C. Extraction of the fresh plant material was carried out as soon as possible after collection. Plants leaves (1 g wet weight per sample) were placed in 3 ml of 80% methanol and ground using a mortar and a pestle. The homogenates were transferred to 25 ml tubes and shaken in the dark at 20°C for 48 h. The homogenates were stored in the dark at -20°C for 48 h to ensure maximum extraction of phenolics (Awika *et al.*, 2003). Samples were equilibrated to room temperature and centrifuged (15 500 x g, 15 min at room temperature) and the supernatant decanted. Each residue was extracted twice (3 ml 80% methanol) and centrifuged (15 500 x g, 5 min at room temperature) until the extracts were clear. The concentrations of the extracts were measured as the actual dry weight of plant material (1 g wet weight dried at 60°C for 12 h) per volume, as described by Halvorsen *et al.* (2002).

4.6.3 Preparation of working reagents for the assay of antioxidant capacity and phenolic content

4.6.3.1 The DPPH˙ reagent

The study investigated the antioxidant activities of different plant extracts using DPPH˙ (25 mg/l), prepared fresh in 80% methanol using 45 ml eppendorf tubes and protected from light. The assay for DPPH˙ scavenging activity was initially developed using an extract from *Carissa haematocarpa*. The stability of the DPPH˙ radical solution was monitored throughout the experiments and the initial absorbance of DPPH˙ was ± 0.600.

4.6.3.2 The $ABTS^{\cdot+}$ reagent

$ABTS^{\cdot+}$ was generated by oxidation of ABTS salts with potassium persulfate (1:0.5, volume/volume (v/v); both were prepared in ddH_2O. A 7 mM ABTS solution was added to 2.45 mM of potassium persulfate. The reaction mixture was left to stand in the dark at room temperature for 12 h before performing the antioxidant assays. Prior to analysis of these extracts, the $ABTS^{\cdot+}$ stock solution was diluted with PBS, pH 7.4, containing 150 mM NaCl and dissolved to an absorbance of 0.700 ± 0.012 at 734 nm. The $ABTS^{\cdot+}$ cation solution was equilibrated at 30°C for 30 min and kept at 4°C until required. The ABTS absorbance was monitored for stability throughout the analysis.

4.6.3.3 The FRAP reagent

The FRAP reagent was prepared daily (for compatibility with the previous analysis) by mixing 300 mM acetate buffer (pH 3.6), 10 mM of 2,4,6-TPTZ solution in 40 mM hydrochloric acid and 20 mM of ferric chloride (Fe_3Cl) solution in proportions of 10:1:1 (v/v/v), respectively. The FRAP reagent was used at 37°C.

4.6.3.4 Folin-Ciocalteu reagent

For the preparation of the standard stock solution, 0.05 g of gallic acid was dissolved in 80% methanol to a final volume of 10 ml and was stored at 4°C. For the preparation of working solutions of gallic acid, the following volumes: 0, 10, 20, 30, 50 and 100 µL were pipetted separately and diluted to a total volume of 1 ml using 80% methanol. A standard calibration curve was constructed using six concentrations: 0, 50, 100, 150, 250, 500 mg/l gallic acid. A 0.2 N Folin-Ciocalteu reagent was prepared

in ddH_2O. Anhydrous sodium carbonate (Na_2CO_3) was prepared as a 20% solution by dissolving 20 g of salt in 80 ml of ddH_2O. The solution was heated for 30 min at 60°C, cooled to room temperature and the volume made up to 100 ml.

4.6.4 Antioxidants and total phenolic assays

4.6.4.1 The DPPH radical scavenging activity assay

The effect of plant extracts on the $DPPH^{\cdot}$ absorbance was estimated following the procedure described by Brand-Williams *et al.* (1995) and Dávalos *et al.* (2003), with modification. Stock solutions from the crude extracts were prepared as 1 mg/ml working solutions in 80% methanol. Separate concentrations in a range between 0 and 1000 µg/ml of an ascorbic acid standard and plant extracts were prepared. Assays were performed in a microtitre plate reader and absorbance read on a Powerwave spectrophotometer (Bio-Tek Instruments, Inc). Briefly, to a 96-well microtitre plate, 5.2 µl of plant extract was added to 194.8 µl of the 25 mg/l of DPPH radical. The decrease in absorbance was measured at 515 nm for 30 min at 1 min intervals at room temperature. The procedure was followed for all plant extracts, a methanol blank and ascorbic acid controls. For each concentration (0, 250, 500, 750 and 1 000 µg/ml) of the samples under the study, the reaction kinetics were plotted and the percentage of the $DPPH^{\cdot+}$ remaining was calculated as follows:

$$\% \ DPPH^{\cdot}_{rem} = [(A_{515\ nm})_{Sample}/(A_{515\ nm})_{Blank}] \times 100$$

where, $(A_{515\ nm})_{Sample}$ is the absorbance of the test sample and $(A_{515\ nm})_{Blank}$ is the absorbance of the methanol blank sample.

4.6.4.2 The ABTS radical cation antioxidant activity assay

The ABTS radical cation decolourization assay was performed, with minor modifications, according to Re *et al.* (1999). The ABTS radical was warmed to 30°C before use. After incubation of 200 µl $ABTS^{\cdot+}$ cation with 2 µl plant extract at varying concentrations between 0 and 1 000 µg/ml, the absorbance reading was taken immediately for 6 min at 30°C using the Powerwave spectrophotometer (Bio-Tek Instruments, Inc.). The ascorbic acid standard was assessed as described above, and ddH_2O was used as a blank. The percent antioxidant activity was calculated using the following equation:

% decolourization = $[(A_0-A_x)/(A_0) \times 100]$,

where, A_0 is the absorbance of the blank solution, and A_x is the absorbance of the test sample (Lima *et al.*, 2005).

4.6.4.3 Ferric reducing antioxidant power (FRAP) assay

The total antioxidant capacity of each plant extract was determined according the original procedure of Benzie and Strain (1996) using the FRAP assay as modified by Wong *et al.* (2006). In brief, 200 µl of FRAP reagent was heated to 37°C, followed by the addition and mixing of 20 µl of ddH_2O and 6.67 µl of plant extract. Absorbance readings were taken immediately at 593 nm. The temperature was maintained at 37°C and the reaction monitored for 30 min at 1 min intervals. A methanolic solution of ferrous sulfate (0.028 - 0.28 mg/l) was used to generate a linear calibration curve. The results were expressed as mg Fe (II)/g of dry weight of plant material. Ascorbic acid was used as a reference standard in the assay.

4.6.4.4 Assays of the total phenolic content of the plant extracts

Total phenolic content was estimated by the Folin-Ciocalteu colourimetric method, based on the procedure of Waterhouse (no date), using gallic acid as the standard phenolic compound. The method in brief was as follows: for each calibration, 20 µl of the standard phenolic compound, plant extracts and the blank reagent were pipetted into separate tubes, and to each tube, 1.58 ml of ddH_2O was added, followed by the addition of 100 µl of Folin-Ciocalteu reagent. The reaction mixtures were gently mixed and incubated at room temperature for 8 min, after which 250 µl of Na_2CO_3 anhydrous (20% solution) was added and mixed gently by pipetting. The reaction solutions were incubated at 40°C for 30 min with continuous shaking at 100 rpm. The absorbance readings of the resulting blue coloured solutions were measured at 765 nm using the Powerwave spectrophotometer. The results were expressed as gallic acid equivalents (GAE)/g dry weight.

4.6.5 Statistical analysis

All experiments were performed in triplicate for separately prepared sample concentrations. The data are expressed as mean ± standard deviations (SD).

4.7 Results

Due to the relatively high sample number, selected results of plants showing high, medium and low antioxidant activities are represented graphically. A complete data set of the different antioxidant assays is tabulated for all plants at a concentration of 1 mg/ml. Three antioxidant assays are reported.

4.7.1 DPPH radical scavenging activity assay

Each plant extract was tested for the free radical scavenging activities against the DPPH radical using 0, 250, 500, 750 and 1 000 µg/ml as working concentrations. A dose-response characteristic profile of three selected methanolic plant extracts of the twenty-five plants investigated on the scavenging of the DPPH$^{\cdot}$ is presented in Fig. 4.2.

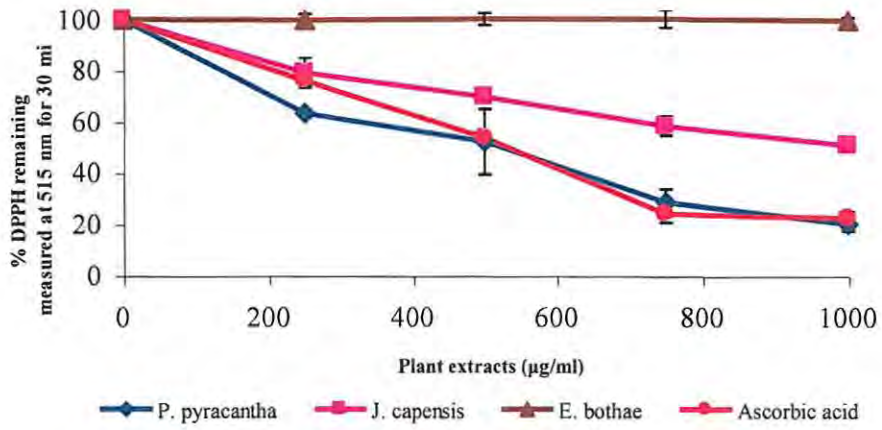


Figure 4.2: Dose-response profiles of three selected plant extracts on the scavenging of DPPH radicals. Ascorbic acid was used as a reference standard. The results are the mean of triplicate determinations ± SD.

The ascorbic acid and *P. pyracantha* had similar scavenging abilities of the DPPH$^{\cdot}$ at 1 000 µg/ml. *P. pyracantha* extracts had higher antioxidant activity relative to other plants investigated, and showed a higher activity at 250 µg/ml compared to ascorbic acid at the same concentration. Similar trends were observed for the other plant species investigated, however, these activities were lower than *P. pyracantha*.

As an example, *O. mucrunata*, is presented in Fig. 4.3 showing the kinetics of DPPH˙ scavenging at the concentrations investigated over 30 min.

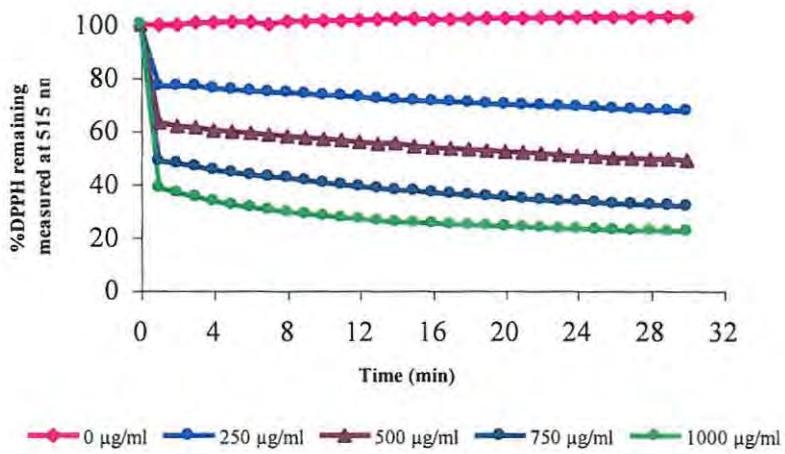


Figure 4.3: Kinetic reaction showing the percent DPPH˙ remaining when monitored for 30 min at different concentrations of *O. mucrunata* extract. The results are the mean of triplicate determinations.

The scavenging of DPPH˙ was most noticeable in the first minute of the assay. The plant extract continued to scavenge the DPPH˙ at a much slower rate, and the reactions were relatively stable after 30 min. A 30 min incubation time was chosen for the DPPH assay for the remaining plants. The scavenging ability was highest at the higher extract concentrations.

The plant antioxidant activities, as determined by the DPPH assay at 1 000 µg/ml, are presented in Table 4.1.

Table 4.1: Percentage DPPH radical remaining after incubation with extracts from various plants reported as part of the diet of black rhinoceroses of the GFRR evaluated at 1 000 µg/ml for 30 min.

Plant family	Plant species	% DPPH˙ remaining
Celastraceae	***Putterlickia pyracantha***	**20.15 ± 2.65**
Anacardiaceae	***Ozoroa mucrunata***	**21.51 ± 4.18**
Euphorbiaceae	***Phyllanthus verrucosus***	**23.43 ± 2.79**
Celastraceae	***Maytenus capitata***	**26.77 ± 1.36**
Ebenaceae	***Euclea undulata***	**33.83 ± 1.64**
Euphorbiaceae	***Jatropha capensis***	**50.83 ± 0.96**
Fabaceae	*Schotia afra*	62.45 ± 1.92
Apocynaceae	*Carissa bispinosa*	63.62 ± 2.37
Celastraceae	*Maytenus heterophylla*	77.50 ± 1.50
Tiliaceae	*Grewia robusta*	77.65 ± 3.76
Anacardiaceae	*Rhus pterota*	82.82 ± 2.29
Apocynaceae	*Carissa haematocarpa*	87.09 ± 4.21
Portulacaceae	*Portulacaria afra*	87.34 ± 2.12
Asteraceae	*Tarchonanthus camphoratus*	89.73 ± 5.10
Salvadoraceae	*Azima tetracantha*	90.38 ± 1.28
Solanaceae	*Lycium ferocissimum*	91.19 ± 2.62
Plumbaginaceae	*Plumbago auriculata*	91.43 ± 0.35
Asteraceae	*Brachylaena ilicifolia*	91.76 ± 4.11
Capparaceae	*Capparis sepiaria*	91.97 ± 1.76
Asparagaceae	*Protasparagus crassicladus*	92.06 ± 1.32
Bignoniaceae	*Rhygozum obovatum*	92.21 ± 3.25
Asparagaceae	*Protasparagus africanus*	93.84 ± 0.50
Boraginaceae	*Ehretia rigida*	94.92 ± 3.02
Asparagaceae	*Protasparagus suaveolens*	95.97 ± 0.63
Euphorbiaceae	*Euphorbia bothae*	99.71 ± 1.30
Positive control	**Ascorbic acid**	**22.34 ± 3.13**

The mean values of triplicate assays ± SD. Plant are listed in the decreasing order of the antioxidant activity. The plants, which scavenged DPPH˙ at ≥ 50%, are shown in bold.

The percentage DPPH$^{\cdot}$ remaining in the reaction mixture ranged from 99.71% to 20.05%. The plants with the highest antioxidant activities were *P. pyracantha* (Celastraceae) > *O. mucrunata* (Anacardiaceae) > *P. verrucosus* (Euphorbiaceae) > *M. capitata* (Celastraceae) > *E. undulata* (Ebenaceae) and *J. capensis* (Euphorbiaceae). The ascorbic acid had a value of 22.34% ± 3.13.

4.7.2 ABTS radical cation decolourization assay

The ability of plant extracts and the ascorbic acid to scavenge the ABTS$^{\cdot+}$ was monitored for 6 min using the concentrations of 0, 250, 500, 750 and 1 000 µg/ml. Fig 4.4 shows the dose-response characteristics of three selected plant extracts.

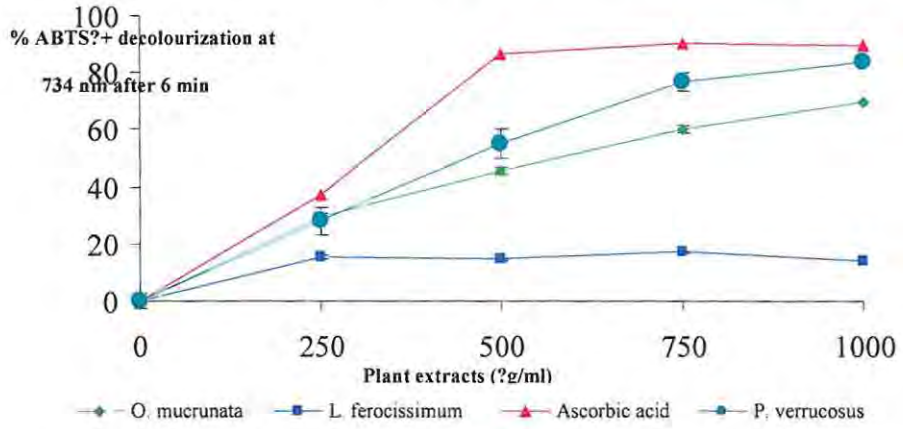


Figure 4.4: Effect of concentration on the decolourization of ABTS$^{\cdot+}$ absorbance by the plant extracts. The reaction was monitored for 6 min and the results are the mean of triplicate determinations.

Plant extracts showed an increasing antioxidant activity with an increase in the concentration, with the ascorbic acid completely scavenging the ABTS$^{\cdot+}$ at 500 µg/ml. The majority of the plant extracts had a limited effect on the scavenging of the ABTS$^{\cdot+}$ (Table 4.2). *O. mucrunata* was used as an example to represent kinetic profiles of antioxidant activities against the ABTS$^{\cdot+}$ when evaluating incubation time at different concentrations as shown in Fig. 4.5.

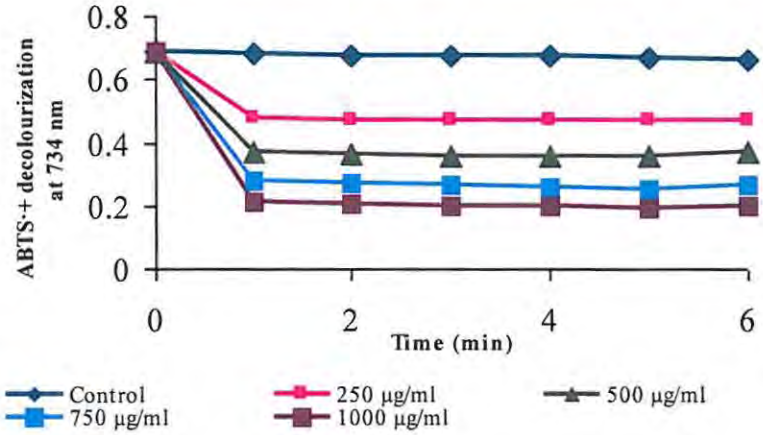


Figure 4.5: Decolourization of $ABTS^{\cdot+}$ by methanolic extracts of *O. mucrunata* when monitored for 6 min with 1 min intervals. The results are the mean of triplicate assays.

Each concentration of the extract reacted similarly with the $ABTS^{\cdot+}$ and completed the reaction in 1 min. The degree of scavenging of the $ABTS^{\cdot+}$ increased with increasing concentration of the extracts.

Three different plant extracts, each showing high, medium and low $ABTS^{\cdot+}$ scavenging activity are presented in Fig. 4.6. The antioxidant activities were evaluated at 1000 µg/ml for 6 min with 1 min intervals.

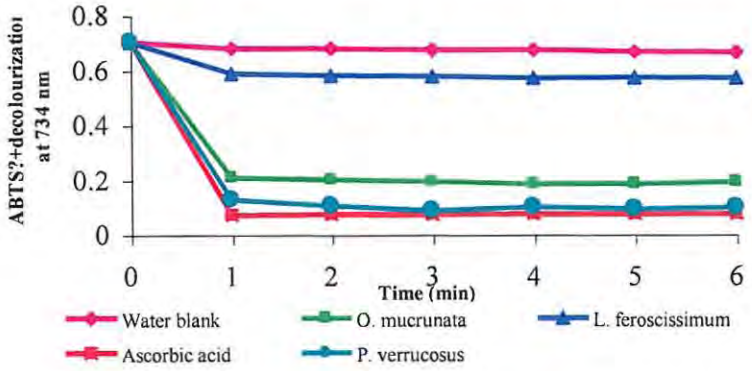


Figure 4.6: Decolourization of $ABTS^{\cdot+}$ absorbance by methanolic extracts of *O. mucrunata, P. verrucosus* and *L. ferocissimum*. Ascorbic acid was used as a positive control and water blank was as a negative control. The results are the mean of triplicate assays.

The percentage decolourization of the $ABTS^{\cdot+}$ absorbance of the twenty-five plants investigated is presented in Table 4.2. Each plant extract (1 000 µg/ml) was evaluated for scavenging activities.

Table 4.2: Percentage of ABTS radical scavenged by various plants reported to be part of the diet of black rhinoceroses of the GFRR evaluated at 1 000 µg/ml for 6 min.

Plant family	Plant species	% ABTS decolourization
Euphorbiaceae	***Phyllanthus verrucosus***	**84.05 ± 3.27**
Celastraceae	***Putterlickia pyracantha***	**80.46 ± 2.15**
Anacardiaceae	***Ozoroa mucrunata***	**70.19 ± 3.62**
Euphorbiaceae	***Jatropha capensis***	**47.26 ± 11.55**
Ebenaceae	***Euclea undulata***	**44.02 ± 6.96**
Celastraceae	***Maytenus capitata***	**41.14 ± 2.85**
Anacardiaceae	*Rhus pterota*	34.26 ± 2.26
Fabaceae	*Schotia afra*	20.17 ± 3.90
Portulacaceae	*Portulacaria afra*	20.03 ± 1.40
Celastraceae	*Maytenus heterophylla*	18.17 ± 3.24
Apocynaceae	*Carissa bispinosa*	17.92 ± 1.99
Asparagaceae	*Protasparagus crassicladus*	14.72 ± 1.92
Tiliaceae	*Grewia robusta*	14.64 ± 1.61
Solanaceae	*Lycium ferocissimum*	14.04 ± 5.26
Asteraceae	*Brachylaena ilicifolia*	10.56 ± 1.70
Plumbaginaceae	*Plumbago auriculata*	10.45 ± 1.98
Cappararaceae	*Capparis sepiaria*	8.53 ± 0.32
Bignoniaceae	*Rhygozum obovatum*	6.38 ± 1.64
Boraginaceae	*Ehretia rigida*	5.75 ± 3.39
Asteraceae	*Tarchonanthus camphoratus*	5.32 ± 3.04
Apocynaceae	*Carissa haematocarpa*	4.68 ± 1.65
Asparagaceae	*Protasparagus africanus*	4.58 ± 0.25
Salvadoraceae	*Azima tetracantha*	4.13 ± 1.65
Asparagaceae	*Protasparagus suaveolens*	4.11 ± 4.17
Euphorbiaceae	*Euphorbia bothae*	2.24 ± 2.01
Positive control	Ascorbic acid	89.3 ± 2.25

The results are mean values of triplicate assays ± SD. Plants are listed in decreasing order of the antioxidant activities. The plants, which scavenged $ABTS^{\cdot+}$ at ≥ 40%, are shown in bold.

The percentage of the $ABTS^{\cdot+}$ scavenged due to antioxidant activities of the plant extracts ranged from 84.05% to 2.24%. Plant extracts showing the strongest antioxidant activities were *P. verrucosus* (84.05 ± 3.27), *P. pyracantha* (80.46 ± 2.15) and *O. mucrunata* (70.19 ± 3.62).

4.7.3 Ferric reducing antioxidant power assay

The plant extracts were also investigated for their ability to reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. A linear calibration curve of ferrous sulfate in the range of 0.028-0.28 mg/l was constructed (Appendix P) to determine the reduction of Fe^{3+}-TPTZ. The ferric reducing potentials were initially measured using different extract concentrations over 30 min, and a representative sample of the plant extracts is shown in Fig. 4.7.

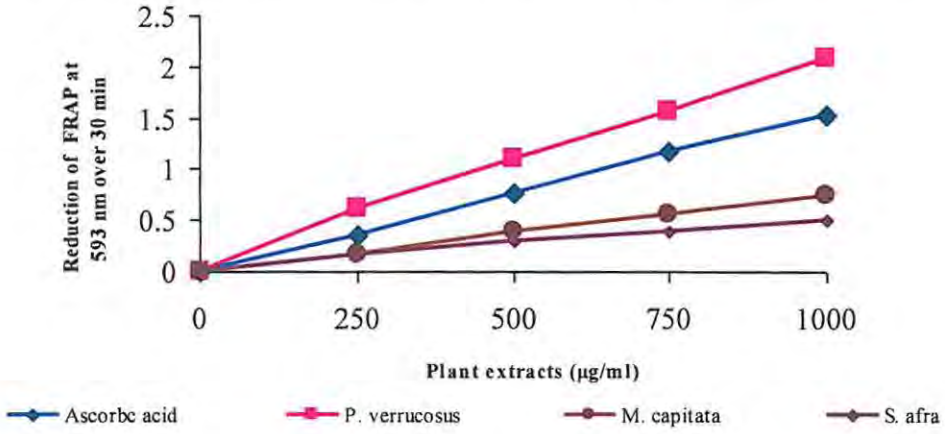


Figure 4.7: Concentration-dependent activities of the three-selected plant extracts on the reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Ascorbic acid was used as the reference standard. The results are the mean of triplicate assays.

The profile shows that an increasing concentration of plant extract leads to greater reduction of the Fe^{3+}-TPTZ. The greatest activity was shown by *P. verrucosus*.

The kinetic reactions of each 1 000 µg/ml plant extract, when monitored at 593 nm for 30 min, are presented in Fig. 4.8.

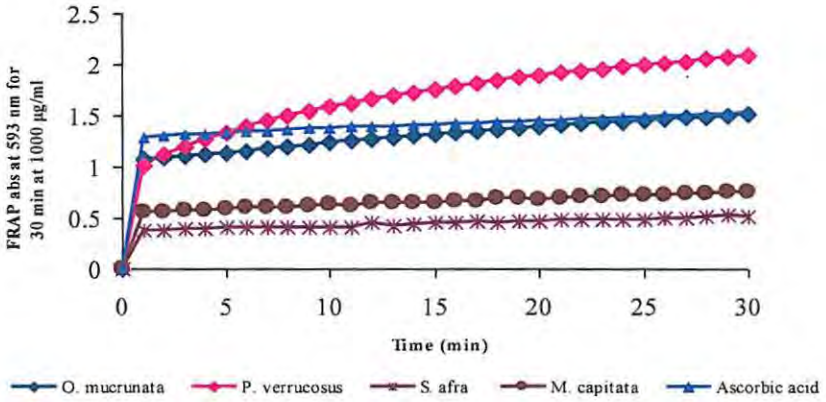


Figure 4.8: Kinetic reactions of each 1 000 µg/ml plant extract on the reduction of the Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex when monitored for 30 min. Ascorbic acid was used as a reference standard. The results are the mean of triplicate assays.

High reduction potential was observed for *P. verrucosus* and the *O. mucrunata*. Both the reactions were time dependent. Ascorbic acid standard completed the reduction reaction rapidly (within 1 min).

All plant extracts reacted rapidly with the FRAP reagent in the first minute, but varied in ferric reducing capacities. Plant extracts of 1 000 µg/ml were used to assess the ferric reducing capacities for 30 min and the results are presented in Table 4.3.

Table 4.3: Ferric reducing antioxidant power (FRAP) assay of GFRR plant extracts evaluated 1 000 µg/ml for 30 min.

Plant family	Plant species	mg Fe (II)/g dry weight
Euphorbiaceae	***Phyllanthus verrucosus***	**62.92 ± 2.50**
Anacardiaceae	***Ozoroa mucrunata***	**45.55 ± 5.97**
Celastraceae	***Putterlickia pyracantha***	**45.13 ± 3.20**
Ebenaceae	***Euclea undulata***	**24.84 ± 1.35**
Celastraceae	***Maytenus capitata***	**23.00 ± 2.01**
Euphorbiaceae	***Jatropha capensis***	**18.89 ± 0.66**
Fabaceae	*Schotia afra*	15.65 ± 0.69
Apocynaceae	*Carissa bispinosa*	14.36 ± 0.13
Celastraceae	*Maytenus heterophylla*	11.12 ± 0.30
Tiliaceae	*Grewia robusta*	8.80 ± 0.85
Anacardiaceae	*Rhus pterota*	7.36 ± 0.36
Asparagaceae	*Protosparagus crassicladus*	5.87 ± 0.84
Bignoniaceae	*Rhygozum obovatum*	5.32 ± 0.88
Asteraceae	*Tarchonanthus camphorates*	4.89 ± 0.59
Asparagaceae	*Protosparagus suaveolens*	4.68 ± 0.54
Capparaceae	*Capparis sepiaria*	4.50 ± 0.62
Apocynaceae	*Carissa haematocarpa*	3.99 ± 0.35
Portulacaceae	*Portulacaria afra*	3.95 ± 0.48
Boraginaceae	*Ehretia rigida*	3.82 ± 0.26
Plumbaginaceae	*Plumbago auriculata*	3.57 ± 0.08
Salvadoraceae	*Azima tetracantha*	3.16 ± 0.82
Asparagaceae	*Protosparagus africanus*	2.97 ± 0.65
Asteraceae	*Brachylaena ilicifolia*	2.27 ± 0.59
Euphorbiaceae	*Euphorbia bothae*	2.05 ± 1.54
Solanaceae	*Lycium ferocissimum*	0.20 ± 0.02
Positive control	Ascorbic acid	46.10± 3.36

The results are represented by the mean triplicate assays ± SD. Plants are listed in decreasing order of Fe^{3+}-TPTZ reducing capacity. Plants with the highest reducing capacity are shown in bold.

The strongest antioxidant capacities were exhibited by *P. verrucosus* > *O. mucrunata* > *P. pyracantha* > *E. undulata* and *M. capitata*.

4.7.4 Total phenolic content of plant extracts

The total phenolic content of the twenty-five plant extracts was studied using the Folin-Ciocalteu assay, and the results are presented in Table 4.4.

A linear calibration curve of gallic acid, in the range between 0 and 500 mg/l was constructed as shown in Appendix Q. There was a wide variation in the total phenolic content of the plant samples investigated, which is shown in Table 4.4. The phenolics ranged from 5.66 to 33.87 GAE/g dry weight. *P. verrucosus* > *J. capensis* > *M. capitata* > *E. undulata* > *C. bispinosa* > *O. mucrunata and P. crassicladus* showed the highest total phenolic content (> 20 GAE/g dry weight).

Table 4.4: Total phenolic content found in various plants reported as part of the diet of the black rhinoceros of the GFRR as determined by the Folin-Ciocalteu assay.

Plant family	Genus and species	GAE/g dry weight
Euphorbiaceae	***Phyllanthus verrucosus***	**33.87 ± 1.73**
Euphorbiaceae	***Jatropha capensis***	**30.97 ± 0.12**
Celastraceae	***Maytenus capitata***	**24.81 ± 1.21**
Ebenaceae	***Euclea undulata***	**24.05 ± 0.18**
Apocynaceae	***Carissa bispinosa***	**23.90 ± 0.77**
Anacardiaceae	***Ozoroa mucrunata***	**22.89 ± 2.29**
Asparagaceae	***Protasparagus crassicladus***	**22.01 ± 0.86**
Anacardiaceae	*Rhus pterota*	19.57 ± 0.84
Fabaceae	*Schortia afra*	17.89 ± 0.46
Apocynaceae	*Carissa haematocarpa*	17.42 ± 0.55
Celastraceae	*Maytenus heterophylla*	17.29 ± 1.21
Plumbaginaceae	*Plumbago auriculata*	16.42 ± 3.39
Asparagaceae	*Protasparagus suaveolens*	15.79 ± 0.75
Bignoniaceae	*Rhygozum obovatum*	15.04 ± 1.46
Asteraceae	*Tarchonanthus camphoratus*	14.33 ± 1.74
Tiliaceae	*Grewia robusta*	14.11 ± 0.70
Euphorbiaceae	*Euphorbia bothae*	13.45 ± 1.06
Celastraceae	*Putterlickia pyracantha*	13.40 ± 1.53
Capparaceae	*Capparis sepiaria*	12.29 ± 1.74
Asteraceae	*Brachylaena ilicifolia*	12.10 ± 0.99
Portulacaceae	*Portulacaria afra*	10.61 ± 1.01
Boraginaceae	*Ehretia rigida*	10.40 ± 1.10
Asparagaceae	*Protasparagus africanus*	10.03 ± 1.41
Salvadoraceae	*Azima tetracantha*	7.65 ± 0.87
Solanaceae	*Lycium ferocissimum*	5.66 ± 0.40

The results are represented as the mean of triplicate assays ± SD. Plants are listed in the decreasing order of the phenolic contents. GAE = gallic acid equivalents (mg/l).

4.7.5 Correlation of antioxidant activities of the twenty-five plant extracts with total phenolic content

The total phenolic content of each plant extract was plotted against their antioxidant activity when investigated using the DPPH, ABTS and FRAP assayed as shown in Fig. 4.10 A, B and C.

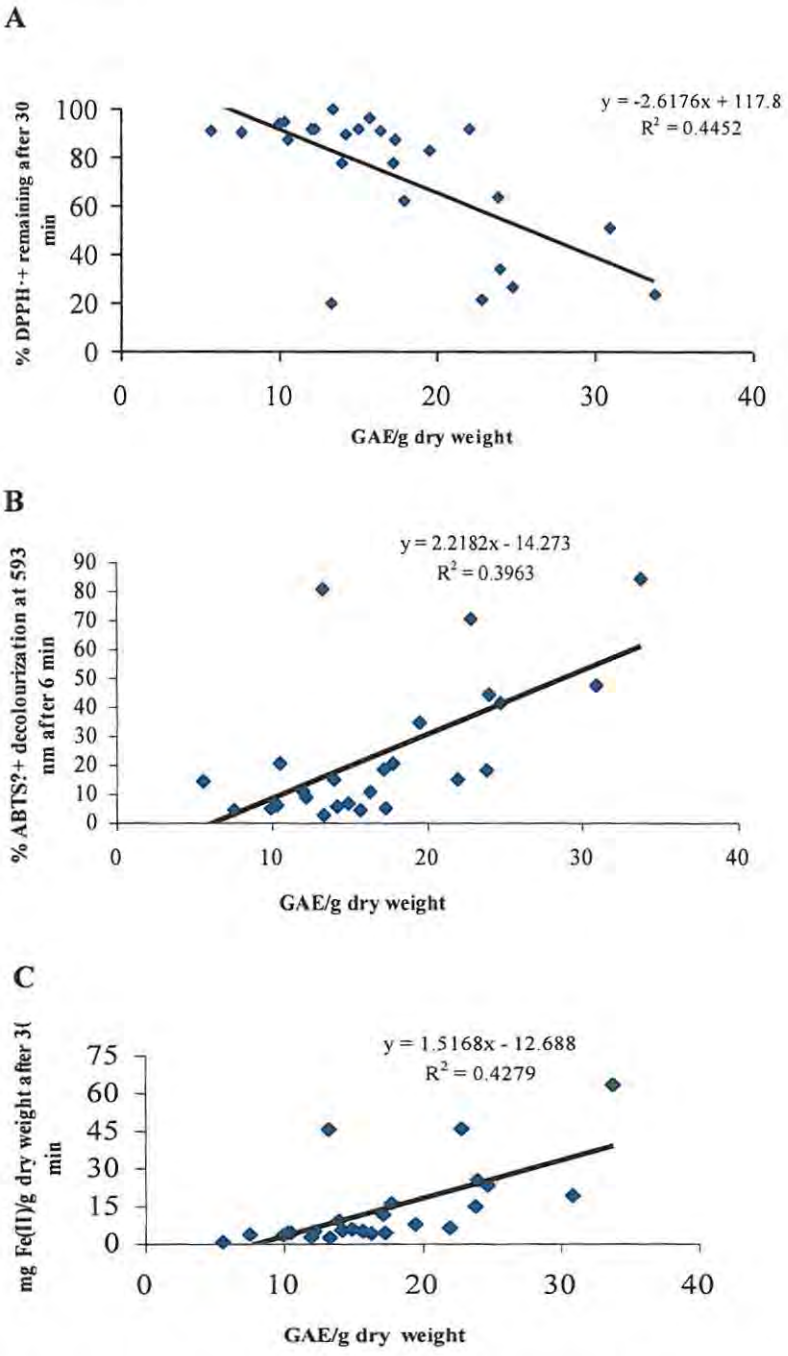


Figure 4.9: Correlation between the total phenolic content and the antioxidant activity of the twenty-five plants evaluated using the A) DPPH, B) ABTS and C) FRAP assays.

The correlation coefficient of the graphs depicted in Fig. 4.9 suggests a low level of correlation between antioxidant activity and phenolic content. However, the graphical representations of the antioxidant assays against total phenolic content indicate that the majority of plants with a low antioxidant activity also have a low phenolic content. Although less pronounced, plants with high antioxidant activities also have relatively high total phenolic content. The exception is *P. pyracantha* which in all three assays had a high antioxidant capacity, but a relatively low total phenolic content of 13.4 GAE/g dry weight.

4.8 Discussion

4.8.1 Preparation of plant extracts

The present study has investigated the antioxidant capacity and the total phenolic content of twenty-five plants of the GFRR, many of which form part of the diet of the black rhinoceros of the GFRR. In order to prepare plant extracts for antioxidant assays, researchers have used different procedures such as freeze-drying or using a rotary evaporator to concentrate plant extracts (Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007). These procedures could result in a loss of active compounds. Furthermore, since antioxidant compounds are water or lipid soluble, or bound to plant cell walls, their recovery depends on the solvent used for extraction (Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007). It is also important to consider how the extraction procedure could influence the qualitative estimation of antioxidant capacities from plant materials (Prakash, 2001; Choi *et al.*, 2007). This is particularly important when different assays are used to measure the antioxidant capacities of plant extracts, as it could result in incorrect interpretation of the antioxidant activity found in the extracts (Prakash, 2001; Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007; Choi *et al.*, 2007).

This study has used a simple methanol extraction as used by Karawita *et al.* (2005) and Wong *et al.* (2006). Extraction from plants such as *E. bothae*, was problematic, and may have led to an underestimation of the antioxidant capacity of these plants.

4.8.2 The DPPH$^{\cdot}$ assay

Free radicals can be produced through normal metabolic processes or by phagocytes, and can be deleterious when produced in large quantities in biological systems. For this reason, antioxidant molecules may be useful in inhibiting the reactivity of these radicals (Cheeseman and Slater, 1993; Martinez-Cayuela, 1995), but the activity of antioxidants against free radicals in biological systems is not entirely clear. As a result, exogenous free radicals have become useful in assessing the antioxidant activities of samples *in vitro* as representative of a biological system (Brand-Williams, 1995; Chen *et al.*, 2006). This has led to a variety of assays to test for the antioxidant

activities of pure compounds and plant extracts using radicals such as DPPH (Chen *et al.*, 2006, Paixão *et al.*, 2007).

Little information is available on the antioxidant capacities of plants forming part of the diet of the black rhinoceros of the GFRR. This study has investigated the antioxidant activities of these plant extracts against the DPPH$^{\cdot}$. The degree of the DPPH$^{\cdot}$ decolourization depends on the nature of antioxidants in the samples (Brand-Williams, 1995) and the stability of DPPH$^{\cdot}$ in reacting with antioxidants in plant extracts was similar to the studies of Brand-Williams *et al.* (1995) and Bondet *et al.* (1997). The present study resulted in using a reaction time of 30 min to allow for any slow reacting antioxidants in the plant extracts to react with the DPPH$^{\cdot}$.

By using a 30 min reaction time, differences in the scavenging capacities of plant extracts against the DPPH$^{\cdot}$ were noticed when assessed in four concentration ranges as shown in Fig. 4.2. Most plants reached the steady state when assayed for 30 min at 1 000 µg/ml shown in Fig. 4.3. Therefore, this concentration was used as the standard concentration for further analysis of radical scavenging activities of plant extracts. The convenience of using one concentration for assessing antioxidant activities of samples is in accordance with the study of Pulido *et al.* (2000) and Mosquera *et al.* (2007).

The percentage of the remaining DPPH$^{\cdot}$, after scavenging by the plant extracts, ranged between 99.71% and 20.05% and demonstrated that if extracts react for 30 min, most reached steady state. Plants having the best antioxidant activities are shown in a decreasing order in Table 4.1. The best six plants were *P. pyracantha* > *O. mucrunata* > *P. verrucosus* > *M. capitata* > *E. undulata* > *J. capensis*. *P. pyracantha* and *O. mucrunata* exhibited higher antioxidant activity than the ascorbic acid at the concentration tested. Among all plant families investigated, two species from the Celastraceae (*P. pyracantha* and *M. capitata*) and two species from Euphorbiaceae (*P. verrucosus* and *J. capensis*) exhibited strong radical scavenging activities. The results suggest that different plant extracts have a variety of antioxidant compounds with different scavenging activities and reaction rates against DPPH$^{\cdot}$. These results are in agreement with the study of Mosquera *et al.* (2007), where plants showing high

scavenging activities against the DPPH radical often were from the family Euphorbiaceae.

The higher antioxidant activity of some plants, when compared to the ascorbic acid at the concentration investigated, would best be explained if compounds in the extracts were isolated and characterized individually. The scavenging activity of the plant extracts is most likely due to the presence of phenolics and flavonoids, which are able to donate hydrogen or electrons. However, detailed mechanisms of the scavenging abilities of plants extracts are not clear and remain for further investigations (Kefalas *et al.*, 2003; Rai *et al.*, 2006). The results obtained in this study suggest that the $DPPH^{\cdot}$ assay is useful in assessing the antioxidant activities of plants browsed by the black rhinoceros.

4.8.3 The $ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ method has been used widely to evaluate the radical scavenging activity of antioxidants of plant extracts as well as pure of compounds. The method is based on the ability of antioxidant molecules to donate hydrogen to the ABTS radical (Miller and Rice-Evans, 1997a and b; Lima *et al.*, 2005; Choi *et al.*, 2007).

The present study investigated the ability of select plants, forming part of the diet of the GFRR black rhinoceros, to scavenge $ABTS^{\cdot+}$. The scavenged $ABTS^{\cdot+}$ is presented as the percentage decrease of absorbance at 734 nm in Fig. 4.4. An example of selected plant extracts showing an increased response to different concentrations over time is shown in Fig. 4.5.

Some studies have experienced a biphasic reaction with the initial reaction being due to the most active compounds reacting rapidly with the radical. The remaining compounds which are less reactive in the sample, give a second slower reaction (Re *et al.*, 1999; Villaño *et al.*, 2004). Due to the plant extracts being relatively crude, and potentially containing a range of unknown antioxidants, this phenomenon may be occurring in these investigations.

The antioxidant activities of the plant extracts against the $ABTS^{\cdot+}$ ranged from 84.05% to 2.24% as shown in Table 4.2. The strongest antioxidant activity was

obtained in the plant extracts from three different families. These were *P. verrucosus* (84.05% ± 3.27), *P. pyracantha* (80.46% ± 2.15) and *O. mucrunata* (70.19% ± 3.62). Most plant extracts contain flavonoids, which may contribute to a wide range of antioxidant activities (Garcia-Alonso *et al.*, 2004; Samarth *et al.*, 2008). According to Shimoi *et al.* (1996), plant flavonoids, which show antioxidant activity *in vitro*, may contribute as antioxidants *in vivo*.

This investigation prepared the $ABTS^{\cdot+}$ in an aqueous solution. Therefore, the hydrophobic compounds in the plant extracts may be underestimated for their antioxidant activities. For this reason, it would be informative to know which compounds contribute to the antioxidant activities. However, the $ABTS^{\cdot+}$ assay gave comparable results to the other antioxidant assays used to assess the antioxidant activities of the selected GFRR plants.

4.8.4 The FRAP assay

The FRAP assay was initially used to test for plasma antioxidant capacity (Benzie and Strain, 1996). This assay was modified to measure the reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex by plant extracts and pure compounds (Pulido *et al.*, 2000; Wong *et al.*, 2006). The ability of antioxidants to increase the absorbance of the FRAP reagent depends on an oxidation-reduction reaction changing the colourless Fe^{3+}-TPTZ to a blue coloured Fe^{2+}-TPTZ complex (Katalinic *et al.*, 2006; Suddhuraju and Becker, 2007). The antioxidant activity is related to the reducing potentials of the test compounds (Firuzi *et al.*, 2005; Paixão *et al.*, 2007).

The present study investigated the ferric reducing antioxidant capacities of GFRR plants. This assay is known to produce conflicting results depending on the solvent used (Pulido *et al.*, 2000). This study standardized the experimental procedure by dissolving the test samples in 80% methanol as described by Firuzi *et al.* (2005).

The plants that showed the strongest ferric reducing capacities were *P. verrucosus* > *O. mucrunata* > *P. pyracantha* > *E. undulata* and *M. capitata*. The ascorbic acid standard showed high ferric reducing capacities when compared to the majority of the plant extracts, but showed less reducing capacity when compared to *P. verrucosus*.

4.8.5 Total phenolic content

This study estimated the total phenolic content in each of the twenty-five plant extracts using the Folin-Ciocalteu assay because phenolic compounds are reported to be good sources of antioxidant activities (Duan *et al.*, 2006; Katalinic *et al.*, 2006). Methanol was used for the phenolic extractions, and for this reason, most compounds extracted in this study were hydrophilic.

Bandoniene and Murkovic (2002) showed that plants contain a diverse group of phenolic compounds such as phenolic acids, hydroxycinnamic acid derivatives and flavonoids. According to Singleton and Rossie (1965), phenolic compounds respond differently in the Folin-Ciocalteu assay due to the number of available phenolic hydroxyl groups that could be oxidized. The total phenolic content obtained from the twenty-five plants investigated ranged from 5.66 to 33.87 GAE/g dry weight. The results are presented in Table 4.4, which indicates seven plants having a phenolic content >20 GAE/g dry weight.

E. undulata, *M. capitata*, *O. mucrunata* and *P. crassicladus* are preferred browse of the black rhinoceros (Brown *et al.* 2003), which had a high phenolic content as shown in the present study. Plants such as *P. verrucosus*, *J. capensis* and *P. pyracantha*, which also had a high phenolic content, have not been reported as preferred browse of the black rhinoceros. Muya and Oguge (2000) reported that the black rhinoceros prefers plants with a low quantity of secondary chemical substances.

It is very difficult to conclude that the obtained values of phenolic compounds in this study are due entirely to the oxidizable phenolic hydroxyl groups present in the plant extracts as certain substances such as chlorophyll, lipids, aromatic amines and sugar derivatives might influence the assays (Singleton and Rossie, 1965; Luximon-Ramma *et al.*, 2002; Dávalos *et al.*, 2003). Furthermore, the influence of the phenolic content found in the preferred diet of the black rhinoceros needs further investigations to determine its effect.

4.8.6 Correlation between antioxidant activities, phenolic content and black rhinoceros browse

Antioxidant studies typically use different analytical methods to determine antioxidant activity due to the differences in the chemical reactions involved in the assays (Paixão *et al.*, 2007). This study has established the *P. verrucosus, O. mucrunata, E. undulata, M. capitata* and *J. capensis* as the best five plants exhibiting high antioxidant activities and phenolic content between the four assays. Extracts exhibiting high antioxidant activities by one method showed good antioxidant activity by the other methods. This is also applicable to plants with low antioxidant activities and phenolic contents when evaluated alone (Fukumoto and Mazza, 2000). An exception is the plant extract *P. pyracantha* which showed high antioxidant activity for all three antioxidant assays, but had a relatively low total phenolic content. *P. crassicladus* and *C. bispinosa* had total phenolic contents of 22.01 and 23.90 GAE/g dry weight, respectively. However, both these plant extracts had low antioxidant activities when assayed by the three antioxidant assays.

Ausland *et al.* (2002) and Brown *et al.* (2003) reported *E. bothae, G. robusta, J. capensis, P. auriculata, A. tetracantha, E. undulata, O. mucrunata* and *R. obovatum* as the preferred plants in the diet of the black rhinoceros. In our study using the *rbc*L gene as a molecular marker, *C. bispinosa* was the most frequently identified plant in the dung. Of the preferred browse, three plant species, namely *J. capensis, E. undulata* and *O. mucrunata* were identified as having high antioxidant activity. The *C. bispinosa* showed a relatively a high total phenolic content when compared to the other plants investigated. However, most of the plants identified as the black rhinoceros browse in the GFRR had low antioxidant and total phenolic content. From the results of this study, and the diet preferences observed by Ausland *et al.* (2002) and Brown *et al.* (2003), it might be considered that the black rhinoceros prefers plants with low antioxidant activities. However, this cannot be stated with any certainty. The black rhinoceros appears to select plants that give it a balanced diet, which may be the reason for the successful propagation of this species on the GFRR.

CHAPTER FIVE
CONCLUSION

A large increase in the number of black rhinoceroses, from only four introduced to the GFRR in 1986, motivated the present study to investigate molecular methods of diet determination, and the evaluation of the nutritional value of their browse. As an attempt to contribute to the conservation and management of the black rhinoceros in the GFRR, this study adopted molecular and biochemical approaches to characterize the diet of the black rhinoceros.

Initially plant DNA was amplified and the *rbc*L gene partially sequenced from a variety of plants collected in the GFRR. These plants were chosen based partly on the results of observational studies of browsing black rhinoceros. The *rbc*L sequences of these plants served as a reference collection from which unknown sequences obtained later in the study could be compared and identified. Difficulties in extracting DNA from certain plants were overcome by using a modified CTAB extraction protocol. The addition of BSA to PCR reaction mixtures allowed for the PCR amplification of extracted DNA samples containing *Taq* polymerase inhibitors.

Of the twenty-three plants sequenced, sixteen were not identified when compared to plant sequences in the GenBank database. The sequences generated in this study will be deposited in the GenBank database once the plant specimens have been lodged with the Selmer Schonland Herbarium in Grahamstown, which will be done in the near future. The 802 bp *rbc*L gene sequence was unable to discriminate between *C. haematocarpa*, *M. nemorosa* and *P. verrucosus*, as these sequences proved to be identical. For the discrimination of these plants, a sequence greater than 1 kb may be required to yield sufficient variation, as suggested by Kress *et al.* (2005).

A proof of concept study of a single dung sample identified *C. bispinosa*, *P. afra, P. crassicladus, P. auriculata*, *R. pterota* and *L. ferocissimum*. Extraction of DNA from dung is challenging as the DNA is often degraded. Extracting a representative sample is difficult to ascertain, especially from a heterogeneous dung sample. However, in this study, the *rbc*L gene from DNA extracted from dung was amplified,

cloned and sequenced. Ideally, when using this approach, a larger number of clones should be sequenced to allow for a more conclusive result. However, this is labour intensive and time consuming, therefore in this study, it was decided to explore GS FLX sequencing.

GS FLX sequencing has the potential to generate a large number of sequences from a single sample. Sequence data was obtained on dung samples collected t four different seasons and *C. bispinosa* was identified as the most prevalent plant in these dung samples. Although GS FLX generated a larger data set, a higher number of sequences were expected. The reason offered for these disappointing results is that the clonal DNA amplification product was too long. In future, primers should be designed to amplify a suitably variable segment of the *rbc*L gene, approximately 450 bp in length.

The main reason for using the *rbc*L gene in this study was that it is plant specific and being a chloroplast gene it is highly conserved, yet has sufficient variation to allow for differentiation between plants. Although the DNA from dung was often degraded, the template was of sufficient quality for PCR amplification of the 802 bp of the *rbc*L gene. Future studies should utilize primers that amplify a shorter segment of DNA, and which covers a more variable region of the *rbc*L gene. It is most likely that this variable region would be on the 3′ end of the gene. It may be necessary also to design primers for specific plants, or families of plants, due to sequence variation between the plants. This would probably lead to investigations using multiplex PCR.

The PCR may have shown bias when amplifying plants from dung samples. For example, *E. bothae* was identified as the preferred browse of the black rhinoceros by Ausland and Sviepe, (2000) and by Brown *et al.* (2003) yet this plant was not identified in the dung samples analyzed by molecular techniques. In contrast, the *C. bispinosa* was identified in all four seasonal dung samples, but was not identified as a significant component of the diet from observation studies. The *C. bispinosa* is tough and woody while *E. bothae* is a succulent plant with soft tissues, making it possible that its DNA did not survive digestion. The identification of succulent soft tissue plants such as *E. bothae* in dung may be a limitation of the molecular approach.

Although certain plants were identified using both observational and molecular techniques, other plants such as *C. bispinosa* were only identified in this molecular study. As these methods identified different plants browsed by the black rhinoceros, it may be valuable to use both of these methods together for the identification of plants in the diet. It may also be of value to identify other plant specific genes or regions of DNA, to allow for an increased ability to differentiate between plants present in the black rhinoceros dung.

Plants identified in the dung or by observation studies of the black rhinoceros were assessed for antioxidant capacities and their total phenolic content. An important finding of this study is illustrated clearly from an investigation of the relative position of certain plant species in Tables 4.1 – 4.3 which present the results of three different antioxidant assays. It is particularly evident that it is the same six plants that have the highest antioxidant activity in each assay and, furthermore, five of these plants also show the highest phenolic content (Table 4.4). This is in spite of each assay being chemically different. Dávalos *et al.* (2003) reported on the antioxidant capacity of seven different commercial dietary antioxidant supplements evaluated by three different methodologies and showed different antioxidant activity patterns, depending on the method, yet a range of samples showed the same overall pattern. Other studies report that antioxidant activities of particular compounds may vary from one study to another, even when the same assay is used making comparative data difficult (Re *et al.*, 1999; Paixao *et al.*, 2007). No one method is entirely suitable for predicting antioxidant capacity of an extract and the use of more than one method is recommended, suggesting the use of caution in the interpretation of results (Luximon-Ramma *et al.*, 2002).

A comparison of the major plant species indicates that only a few plants with high antioxidant capacity and phenolic content are favoured by the black rhinoceros. The black rhinoceros appear to prefer plants of relatively low antioxidant and total phenolic content. However, based upon these studies alone, it would be premature to suggest that the black rhinoceros selects plants for its diet based mainly on these criteria.

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APPENDICES

Appendix A: Isolation of genomic DNA from plant tissue using the DNeasy Plant Mini Kit

Plant material (0.1 g) was ground to a fine powder with liquid nitrogen. The material was placed into a safe lock microcentrifuge tube containing 400 µl AP1 buffer, followed by the addition of 4 µl RNase A. The mixture was incubated for 10 min at 65°C, followed by mixing (x 3) between incubation by inversion. Thereafter, 130 µl AP2 buffer was added to the lysate, followed by incubation for 5 min on ice. The lysate was applied to the QIAshredder Mini Spin Column which was placed in a 2 ml collection tube, and centrifuged (15 500 x g, 2 min at room temperature). The fraction collected was transferred to a new microcentrifuge tube without disturbing the pelleted cells. A 1.5 volume of AP/3 buffer added to the recovered lysate and was mixed immediately by pipetting. A 650 µl of the lysate was pippeted into the DNeasy Mini Spin Column set in a collection tube. The mixture was centrifuged (6 000 x g, for 1 min at room temperature) and the recovered eluent was discarded. The column was placed in a new 2 ml collection tube and 500 µl AW buffer was added. The tube was centrifuged (6 000 x g, 1 min at room temperature). The eluent was discarded and the collection tube was reused. A further 500 µl AW buffer was added to the DNeasy Mini Spin Column, followed by centrifugation (15 500 x g, 2 min at room temperature). The column was centrifuged (15 500 x g, 5 min at room temperature) to dry the membrane. The column was transferred to a 1.5 ml microcentrifuge tube and 100 µl ddH_2O was added directly to the DNeasy membrane. The tubes were incubated for 5 min at room temperature and centrifuged for 1 min at 6 000 x g to elute genomic DNA. The eluent was stored at –20°C.

Appendix B: Composition of buffers and solutions

Appendix B1: Tris (1 M), pH 8 per litre

Tris base salt (121.2 g) was added to 700 ml $dddH_2O$ and the pH of 8 was adjusted with HCl. The solution was made to a litre with $dddH_2O$ and autoclaved for 20 min.

Appendix B2: 0.5 M EDTA, pH 8.0 per 200 ml

EDTA salt (37.2 g) was added in 100 ml $dddH_2O$ and the pH was adjusted with 5 M NaOH. The solution was made up to 200 ml with $dddH_2O$ and autoclaved prior to use.

Appendix B3: 50 X TAE (Tris-Acetate-EDTA) buffer, pH 8, per litre

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml

Appendix B4: CTAB extraction buffer

2% CTAB

1.4 M NaCl

20 mM EDTA

100 mM Tris buffer, pH 8

2% PVP

2% BSA

Appendix C: PCR mixture for the amplification of the *rbc*L (802 bp) gene in a total of 15 µl

Reagents	Final Concentration	Quantity (vol)
Triple deionized water		Variable
5 X *Taq* Buffer	1X	3 µl
10 mM dNTP mix	200 µM each	0.3 µl
25 mM $MgCl_2$	1.4 mM	0.84 µl
Genomic DNA template	200 ng DNA/15 µl	X (Variable)
Forward primer *rbc*L 10 µM	0.8 µM	1.2 µl
Reverse Primer *rbc*L 10 µM	0.8 µM	1.2 µl
Taq Polymerase 5U/ µl	1.0 U/µl	0.2 µl
BSA 20 mg/ml	2.4 – 2.7 mg/ml	1.8 - 3 µl

Appendix D: BSA addition to the extraction of DNA of the listed plants

Plant species	**BSA (µg)**
Azima tetracantha *Euclea undulata*	39
Phyllanthus verrucosus *Mayetenus nemorosa*	69

Appendix E: Preparations of chemically competent cells, JM 109 strain

To maintain lab stock of highly efficient low background *Escherichia coli* strain, JM 109 competent cells for plasmid transformations was prepared as follows:

Appendix E1: Time required for preparing competent cells

Day 1: Cells were plated and incubated at 37°C overnight.

Day 2: Colonies were selected and cultured for 4 hours at 37°C until the OD reached between 0.6 and 0.8.

Appendix E2: Preparations of reagents

The KCl of 3 M was prepared in 50 ml; 1M MnCl in 50 ml; 1M CaCl in 50 ml. The CH_3COOK 30 mM, pH 5.8, 10 mM MOPS, pH 6.8 and 15% m/v glycerol was prepared in 500 ml using $dddH_2O$.

Appendix E3: Buffer 1: RF1, pH 5.8

The 90 mM KCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 30 mM CH_3COOK, pH 5.8; 15% m/v glycerol per 100 ml total volume.

Approximately, 30 mM CH_3COOK and 15 % m/v glycerol were mixed and the pH was adjusted to 5.8 with HCl and autoclaved prior to addition of 90 mM KCl, 50 mM $MnCl_2$ and 10 mM $CaCl_2$, respectively. The mixture was then stored at 4°C until required.

Appendix E4: Buffer 2: RF2, pH 6.8

MOPS 10 mM, pH 6.8; 15 % m/v Glycerol; 10 mM KCl; 75 mM $CaCl_2$ per 100 ml. Approximately, 10 mM MOPS, 15 % m/v glycerol were mixed in a 100 ml clean reagent bottle and the pH was adjusted to 6.8 with KOH and autoclaved prior to addition of 10 mM KCl and 75 mM $CaCl_2$ respectively. The solution was stored in 4°C until required.

Appendix E5: Procedure for preparing competent cells

Step 1: *E. coli* JM 109 strain cells, stored at -80°C were streaked on a LB agar plates (without any selective agent) and incubated at 37°C for 16 h to isolate colonies.

Step 2: Using a sterile inoculating loop, a single colony was scraped from the agar surface, inoculated and grown in a sterile 5 ml LB broth medium (without a selective antibiotic since these cells do not contain plasmid) and incubated at 37°C with vigorous shaking at 180 rpm for 12 h using a Multishaker PSU 20, BOECO (Germany)

Step 3: Cells were diluted 1:200 in sterile LB broth and were grown at 37°C on a 180 rpm shaker until they reach an OD between 0.6 and 0.8 (approximately 4 h). The cells were quantified using Biowave spectrophotometer at 600 nm by making 1:5 dilutions in 1 ml cuvette.

Step 4: The cells were cooled for 10 min in an ice bath prior to processing. The cells were pelleted into four separated pre-chilled 25 ml sterile centrifuge bottles and were centrifuged for 10 min at 2 300 x g at 4°C using the Beckman Avanti centrifuge (JA-20 rotor).

Step 5: The supernatant was decanted and the bacterial pellets were gently resuspended in 1/200-culture volume of ice-cold RF1 solution on ice, making sure that less than 5 min was taken for this procedure. These suspensions were kept for 20 min on ice prior to centrifugation at 2 300 x g at 4°C for 10 min.

Step 6: The supernatant was decanted and the bacterial pellets were gently resuspended in 1/400 culture volume of ice-cold RF2 solution on ice and were dispensed in multiple 100 µL aliquots using pre-chilled eppendorf tubes. The chemically competent cells were then stored at -70°C freezer until required.

Appendix F: Media and plates

Appendix F1: Luria Bertani (LB) plates with Ampicillin

The LB medium prepared was supplemented with the agar powder (15 g) to a litre using $dddH_2O$. The medium was autoclaved and allowed to cool to 50°C before adding 100 µg/ml ampicillin. Ready-made *E. coli* FastMedia LB Agar IPTG/X-Gal was also used as alternative in the preparations of plates.

Appendix F2: LB plates with Ampicillin/IPTG/X-Gal

The LB plates were was supplemented with 0.5 mM IPTG and 80 µg/ml X-Gal. Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal was spread over the surface of the LB-ampicillin plates and was allowed to absorb for 30 minutes at 37°C prior to use. Alternatively, the ready-made *E. coli* FastMedia LB Liquid Amp was used for preparation of plates.

Appendix F3: LB medium per litre or Ready made LB medium

Bacto®-Tryptone (10 g), 5 g Bacto®- Yeast Extract and 5 g NaCl were dissolved in a litre of $dddH_2O$. The pH was adjusted to 7.0. Ready-made LB medium was also used as an efficient media for bacterial growth.

Appendix F4: SOC (Super Optimized Culture) medium in 100 ml

The Bacto®-Tryptone (2 g), 0.5 g Bacto®-Yeast Extract, 1 ml of 1 M NaCl and 0.25 ml 1 M KCl were dissolved in 97 ml $dddH_2O$ and autoclaved. The medium was allowed to cool at room temperature and later, 1 ml of 2 M Mg^{2+} and glucose stock solutions were each added to a final concentration of 20 mM, followed by the addition of 1 ml $dddH_2O$. The pH of the solution was adjusted to 7.0 and was kept at 4°C until used. This media was used for growth and recovery of *E. coli* cells after transformation.

Appendix G: Transformations

High efficiency JM109 competent cells prepared and stored frozen as described in Appendix E were thawed for 5 min in an ice bath. In brief, 50 µl of the cells were carefully transferred into 1.5 ml microcentrifuge tubes containing 2 µl of ligation reaction mixture. The tubes were gently agitated and incubated on ice for 20 min for binding. The transformation mixture was heat-shocked for 35s in a heating block at 42°C for permeation of the plasmid DNA into the cells. The tubes were immediately placed on ice and incubated for 2 min. Super Optimized Culture (SOC) medium at room temperature was added (950 µl) to both tubes containing transformants for recovery and were further incubated for 45 min at 37°C with vigorous shaking at 180 rpm. The tubes were microcentrifuged at 6 000 x g for 1 min to collect the cells. Each transformation culture of 100 µl was plated separately into a selective antibiotic plate as prepared in Appendix F1 and F2. The plates were incubated for 12 h at 37°C for colony growth.

Appendix H: Plasmid "Easy" Preparation

Preparations of easy buffer in 50 ml total volume are listed below

1 M Tris-Cl Stock pH 8	0.5 ml
0.5 M EDTA stock pH 8	0.1 ml
15% w/v Sucrose (weigh out fresh)	7.5 g
10 mg/ml RNAse A stock (DNAse free)	1 ml
100 mg/ml lysozyme	1 ml

The buffer was filter sterilized and stored at –20°C.

Procedure

Overnight cultures of approximately 1.5 ml were microcentrifuged in 1.5 ml sterilized centrifuge tubes for 2 min at 7 400 x g using a benchtop spectrafuge 24D (Labnet International, Inc). The supernatants were decanted and 50 ul of easy buffer prepared was added to the pelleted bacterial cells for resuspension. The cells were incubated at 37°C for 30 min and boiled for 1 min at 100°C. The tubes were immediately incubated on ice for 5 min and microfuged for 10 min at 15 500 x g. The supernatants withdrawn were used in PCR for screening of the *rbc*L insert.

Appendix I: Purification of plasmid DNA from *E. coli* cultures using a QIAprep Spin Miniprep Kit

Overnight cultures of *E. coli* in LB medium were pelleted for 2 min at 7 400 x g. The recovered medium was discarded and the pelleted bacterial cells were resuspended in 250 µl of P1 buffer. 250 µl of P2 buffer was added to the bacterial mixtures and the tubes were gently inverted two to three times. After addition of 350 µl N3 buffer, the tubes were immediately mixed two to three times by inversion. The tubes were centrifuged for 10 min at 15 500 x g in a table-top microcentrifuge. The supernatant recovered from each tube was applied to the QIAprep Spin Column by adding 0.5 ml PB buffer and centrifuged for 1 min at 15 500 x g. The eluate was discarded and the columns were placed in new microcentrifuged tubes. The columns were washed by adding 0.75 ml PE buffer and centruged for 1 min at 6 000 x g. The eluate was discarded and additional centrifugations were carried out to all tubes to remove the residual buffer. The columns were further placed in new sterile 1.5 ml microcentrifuge tubes, followed by addition of 50 µl sterile ddH_2O. The tubes were left to stand at room temperature for 1 min before elution of the plasmid DNA by centrifugation for 1 min at 6 000 x g. The plasmid DNA was stored at –20°C required.

Appendix J: Plasmid polymerase chain reaction mixture for the screening of *rbc*L gene inserts using M13 primers

Reagents	Final Concentration	Quantity in 15 µL
Triple deionized water		Variable
5 X *Taq* Buffer	1X	3 µl
10 mM dNTP mix	200 µM each	0.3 µl
25 mM $MgCl_2$	1.4 mM	0.84 µl
Plasmid DNA	200 ng/15 µl	1.2 µl
Forward primer M13 10 µM	0.8 µM	1.2 µl
Reverse Primer M13 10 µM	0.8 µM	1.2 µl
Taq Polymerase 5U/µl	1.0 U/µl	0.2 µl

Appendix K: ClustalW 2.0.8 multiple sequence alignment of plants from the GFRR

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P.afra              ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCAGGTGTTAAAGATTAC 60
G.robusta           ATGTCACCACAAACAGAGACTAAAGCTTTTGTTGGATTCAAAGCTGGTGTTAAAGATTAC 60
O.mucrunata         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGCGTTAAAGACTAT 60
R.pterota           ATGTCACCACAAACAGAGACTAAAGCACATGTTGGATTCAAAGCCGGCGTTAAAGACTAT 60
P.auriculata        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAT 60
A.tetracantha       ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAT 60
E.bothae            ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
J.capensis          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
G.capitata          ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGCGTTAAAGATTAT 60
M.capitata          ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
P.pyracantha        ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGCGTTAAAGATTAT 60
C.rudis             ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
C.haematocarpa      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
M.nemorosa          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
P.verrucosus        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
C.bispinosa         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
S.myrtina           ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGTAC 60
L.ferocissimum      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGTAC 60
B.ilicifolia        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAT 60
E.undulata          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAC 60
P.crassicladus      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
P.africanus         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
P.suaveolens        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
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P.afra              AAATTGACTTATTATACTCCTGAATATCAACCTCAGGATACTGATATCTTGGCAGCATTC 120
G.robusta           AAATTGACTTATTATACTCCTGATTATCAAACCCTAGATACTGATATCTTGGCAGCATTT 120
O.mucrunata         AAATTGACTTATTATACTCCTGACTATATAACCAAAGATACTGATATCTTGGCAGCATTC 120
R.pterota           AAATTGACTTATTATACTCCTGAGTATATAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.auriculata        AAATTGACTTATTATACTCCTGAGTATCAAGTCAAAGATACTGATATCTTCGCGGCCTTC 120
A.tetracantha       AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATTTTGGCAGCATTC 120
E.bothae            AAATTGACTTATTATACTCCTGAATATCAAACCAAAGATACTGATATCTTCGCAGCATTC 120
J.capensis          AAATTGACTTATTATACTCCTCAGTATCAAACCAAAGATACTGATATCTTGGCAGCATTC 120
G.capitata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
M.capitata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.pyracantha        AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
C.rudis             AAATTGACTTATTACACTCCTGACTATGAAACCAAAGATACCGATATCTTGGCAGCATTT 120
C.haematocarpa      AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
M.nemorosa          AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
P.verrucosus        AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
C.bispinosa         AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
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L.ferocissimum      AAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACTGATATATTGGCAGCATTC 120
B.ilicifolia        AAATTGACTTATTATACTCCTGACTATCAAACCAAGGATACTGATATCTTGGCAGCATTT 120
E.undulata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.crassicladus      AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.africanus         AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.suaveolens        AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
                    * ********** ******** **  *  ***** ***** ***** ** **

P.afra              CGAGTAACTCCTCAACCTGGAGTTCCGTCAGAAGAAGCAGGGGCCGCAGTAGCTGCCGAA 180
G.robusta           CGAGTAACTGCTCAACCTGGAGTTCCACCAGAGGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
O.mucrunata         CGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGGGCTGCGGTAGCTGCGGAA 180
R.pterota           CGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGGGCTGCGGTAGCTGCGGAA 180
P.auriculata        CGAGTAACTCCTCAACCCGGAGTTCCGCCTGAGGAAGCAGGAGCCGCGGTAGCTGCTGAA 180
A.tetracantha       CGAGTAACTCCTCAACCCGGAGTTCCACCTGAAGAGGCAGGGGCTGCGGTAGCTGCTGAA 180
E.bothae            CGAGTAAGTCCTCAACCTGGAGTTCCACCTGACGAAGCAGGAGCTGCGGTAGCTGCTGAA 180
J.capensis          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAGGAAGCAGGAGCTGCGGTAGCTGCTGAA 180
G.capitata          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
M.capitata          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
P.pyracantha        CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
C.rudis             CGAGTAACTCCTCAACCCGGAGTTCCACCTGAGGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
C.haematocarpa      CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
M.nemorosa          CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
P.verrucosus        CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
C.bispinosa         CGAGTAACTCCTCAACCCCGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
S.myrtina           CGAGTAACTCCTCAACCGGGAGTTCCACCTGAAGAAGCGGGGCCGCGGTAGCTGCCGAA 180
L.ferocissimum      CGAGTAACTCCTCAACCTCGAGTTCCACCTGAAGAAGCAGGGGCCGCGGTAGCTGCGGAA 180
B.ilicifolia        CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCCGCAGTAGCTGCCGAA 180
E.undulata          CGAGTAACTCCTCAACCTGGAGTTCCACCGGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
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P.crassicladus      CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCGCTGCGGTAGCTGCCGAA 180
P.africanus         CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCGCTGCGGTAGCTGCCGAA 180
P.suaveolens        CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCCCTGCGGTAGCTGCCGAA 180
                    ******* * ******* ******** * ** ** ** ** ** ** ******** ***

P.afra              TCTTCTACTGGTACATGGACAACTGTATGGACCGACGGACTTACCAGTCTTGATCGTTAC 240
G.robusta           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
O.mucrunata         TCTTCTACTGGTACATCGACAACTGTGTGGACCGACGGGCTTACCAGCCTTGATCGTTAC 240
R.pterota           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
P.auriculata        TCTTCTACTGGTACATGGACAACCGTGTGGACCGATGGGCTTACCAGCCTTGGTCGTTAC 240
A.tetracantha       TCTTCTACTGGTACATGGACAAGTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
E.bothae            TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAT 240
J.capensis          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAT 240
G.capitata          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
M.capitata          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
P.pyracantha        TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
C.rudis             TCTTCTACTCGTACATGGACAACTGTATGGACTGACGGGCTTACCAGTCTTGATCGTTAC 240
C.haematocarpa      TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
M.nemorosa          TCTTCTACTGGTACATCGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
P.verrucosus        TCTTCTACTGGTACATCGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
C.bispinosa         TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
S.myrtina           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
L.ferocissimum      TCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
B.ilicifolia        TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACGAGCCTTGATCGTTAC 240
E.undulata          TCTTCTACTGGTACATGGACAGCTGTGTGGACCGATGGACTTACTAGTCTTGATCGTTAC 240
P.crassicladus      TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
P.africanus         TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
P.suaveolens        TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
                    ********************** ** ***** ** ** ***** ** **** ******

P.afra              AAAGGACGATGCTACCACATTGATGCCGTTCCTGGAGAAGACAATCAATATATTTGTTAT 300
G.robusta           AAAGGACGATGCTACCACATCGAGCCTGTTCCTGGAGAAGAAAGTCAATTTATTGCTTAT 300
O.mucrunata         AAACGACGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
R.pterota           AAAGGACCATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
P.auriculata        AAAGGGCGATGCTACAACATCGAGCCTGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
A.tetracantha       AAAGGACGATGCTACCACATTGAGCCTGTTCTTGGAGAAGAAAATCAATATATTGCTTAT 300
E.bothae            AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTAT 300
J.capensis          AAAGGACGATGCTACGACATCGAGCCCGTTGCTGGAGAACAAAATCAATATATTGCTTAT 300
G.capitata          AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATTTATTGCTTAT 300
M.capitata          AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAGTCAATTTATTGCTTAT 300
P.pyracantha        AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGACAGAAAAATCAATTTATTGCTTAT 300
C.rudis             AAAGGTCGATGCTACCACATCCAGCCCGTTGCTGGAGAAGAAACTCAATTTATTGCTTAT 300
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M.nemorosa          AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
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C.bispinosa         AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
S.myrtina           AAAGGTCGATGCTATCACATCGAGCCAGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
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B.ilicifolia        AAACGCCCATGCTATGGAATCGAGCCCGTTCCTGGAGAAGAAAATCAATATATTGCTTAT 300
E.undulata          AAAGGGCGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTAT 300
P.crassicladus      AAAGGACGATGCTACCACATCGAGGCCGTTATTGGGGAAGAAAATCAATTTATTGCTTAT 300
P.africanus         AAAGGACGATGCTACCACATCGAGCCCGTTGTTGGGGAAGAAAGTCAATTTATTGCTTAT 300
P.suaveolens        AAAGCACGATGCTACCACATCGAGGCCGTTGTTGGGGAAGAAACTCAATTTATTGCTTAT 300
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P.afra              GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATT 360
G.robusta           GTAGCTTACCCATTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATT 360
O.mucrunata         GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
R.pterota           GTAGCTTACCCTTTAGACCTTTTTCAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.auriculata        GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
A.tetracantha       GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
E.bothae            GTAGCTTACCCCTTAGACCTTTTTGAAGAACGTTCTGTTACTAACATGTTTACCTCCATT 360
J.capensis          GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
G.capitata          GTAGCTTATCCTTTAGACCTTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
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P.pyracantha        GTAGCTTATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
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P.crassicladus      GTAGCTTATCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
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P.suaveolens        GTACCTTATCCTTTAGACCTTTTTGAACAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
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O.mucrunata         GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATC 420
R.pterota           GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATC 420
P.auriculata        GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTGCGAATC 420
A.tetracantha       GTGGGTAATGTATTTGGTTTCAAAGCCCTGCGCGCTCTACGCCTAGAGGATTTGCGAATC 420
E.bothae            GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCGCTACGTCTGGAGGATTTGCGAATC 420
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M.capitata          GTGGGTAATGTCTTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATC 420
P.pyracantha        GTGCGTAATGTATTTGGGTTCAAAGCCCTACCGCCTCTACGTCTGGAAGATTTGCGAATC 420
C.rudis             GTGGGTAATGTATTTGCGTTCAAGGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATC 420
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C.bispinosa         GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
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B.ilicifolia        GTAGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACGTCTGGAAGATTTGCGAATC 420
E.undulata          GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAAGATTTGCGAATC 420
P.crassicladus      GTGGGTAATGTATTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420
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R.pterota           CCTACCGCGTATACAAAAACTTTCCAAGGACCACCGCATGGGATCCAAGTTGAGAGAGAT 480
P.auriculata        CCTCCTGCTTATATTAAAACTTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAAAGAGAT 480
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E.bothae            CCTACTTCTTATACTAAAACTTTCCAAGGGCCACCTCATGGAATCCAAGTTGAGAGAGAT 480
J.capensis          CCTACTGCTTATACTAAAACTTTCCAAGGGCCGCCTCATGGTATCCAAGTTGAGAGAGAT 480
G.capitata          CCCCCTGCTTATTCTAAAACTTTCCAAGGCCGCCGCATGGTATCCAAGTTGACAGAGAT 480
M.capitata          CCCCCTGCTTATTCTAAAACTTTCCAAGGCCCGCCGCATGGTATCCAAGTTGAGAGAGAT 480
P.pyracantha        CCCCCTGCTTATTCTAAAACTTTCCAAGGCCCGCCGCATGGTATCCAAGTTGAGAGAGAT 480
C.rudis             CCCCCTGCTTATACTAAAACTTTCCAAGGCCCGCCTCATGGCATCCAAGTTGAGAGAGAT 480
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S.myrtina           CCTACTTCTTATATTAAAACCTTCCAAGGGCCGCCCCATGGCATCCAAGTTGAGAGAGAT 480
L.ferocissimum      CCTACTGCTTATGTTAAAACTTTCCAAGGTCCGCCTCATGGGATCCAAGTTGAAAGAGAT 480
B.ilicifolia        CCTACTGCGTATGTTAAAACTTTCCAAGGTCCGCCTCACGGCATCCAAGTTGAGAGAGAT 480
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P.verrucosus        AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
C.bispinosa         AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
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L.ferocissimum      AAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
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E.undulata          AAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAACCGAAATTGGGGTTA 540
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P.africanus         AAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAAAATTGGGATTA 540
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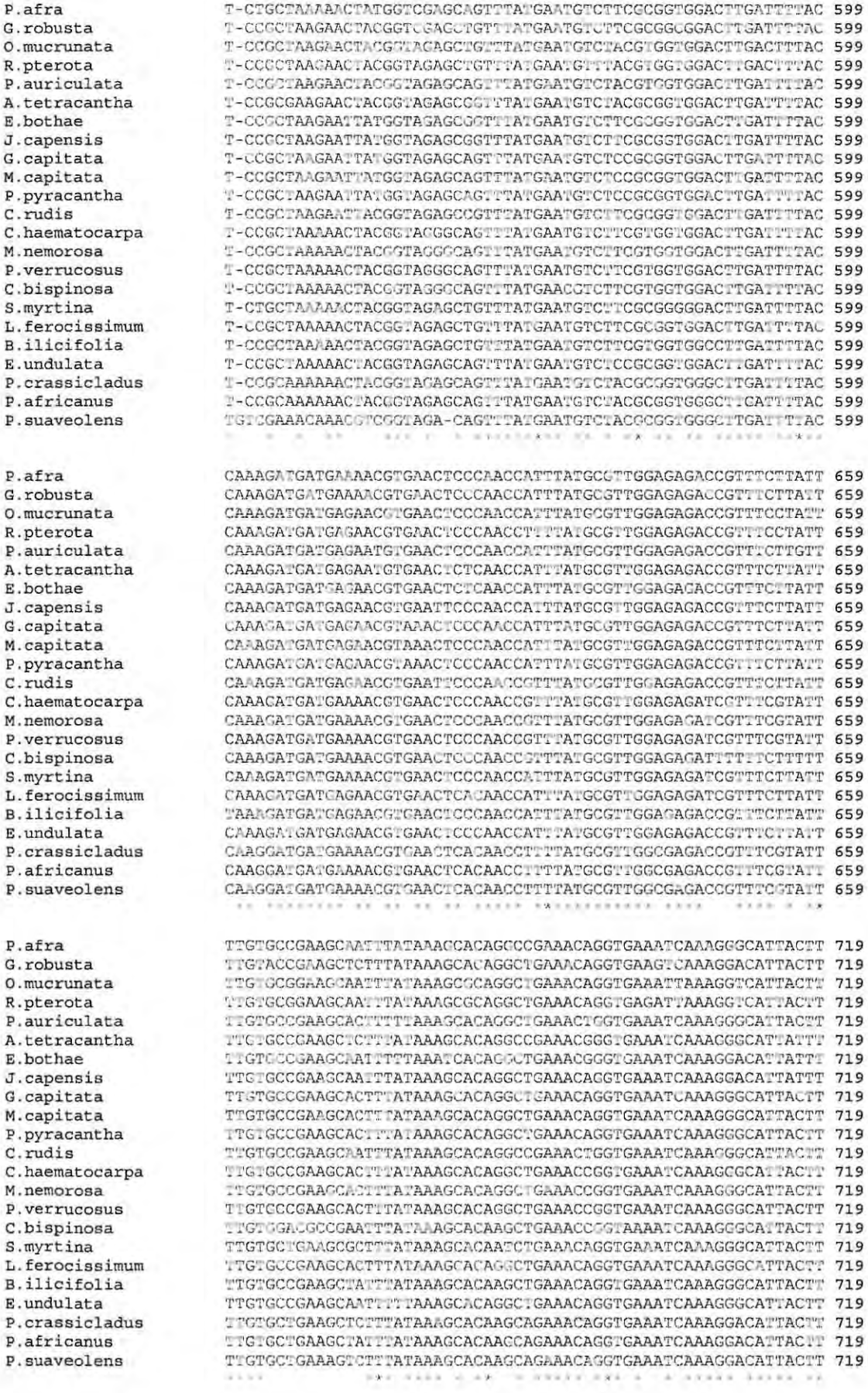
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M.capitata T-CCGCTAAGAATTATGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
P.pyracantha T-CCGCTAAGAATTATGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
C.rudis T-CCGCTAAGAATTACGGTAGAGCCGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
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L.ferocissimum T-CCGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
B.ilicifolia T-CCGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGTGGTGGCCTTGATTTTAC 599
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J.capensis CAAACATGATGAGAACGTGAATTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
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A.tetracantha       GAATGCTACTGCGGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCCAGAGAATT 779
E.bothae            GAATGCTACTGCAGGTACATCCGAACAAATGATCAAAAGGGCTGTATTTGCCAGGGAATT 779
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M.capitata          GAATGCTACTGCCGGTACATGCGAAGAAATGATCAAAAGGGCTGTATTTGCTAGAGAACT 779
P.pyracantha        GAATGCTACTGCCGGTACATGCGAAGAAATGATCAAAAGGGCTGTATTTGCTAGAGAACT 779
C.rudis             GAATGCTACGGCAGGTACATGGGAAGATATGCTCAAAAGGGCTGTATGTGCCAGAGAATT 779
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E.undulata          GAATGCTACTGCAGGTACATGTGAAGAAATGATAAAAAGGGCTGTATTTGCCAGAGAATT 779
P.crassicladus      GAATGCAACTGCAGGTACATGTGAAGAAATGATGAAAAGGGCCGTATTTGCCAGAGAATT 779
P.africanus         GAATGCAACTGCAGGTACATGTGAAGAAATGATTAAAAGGGCCGTATTTGCCAGAGAATT 779
P.suaveolens        GAATGCAACTGCAGGTACATGTGAAGAAATCATGAAAAGGGCCGCATTTGCCAGAGAATT 779
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R.pterota           GGGAGTTCCTATCGTAATGCATG 802
P.auriculata        GGGAGTTCCTATCGTAATGCATG 802
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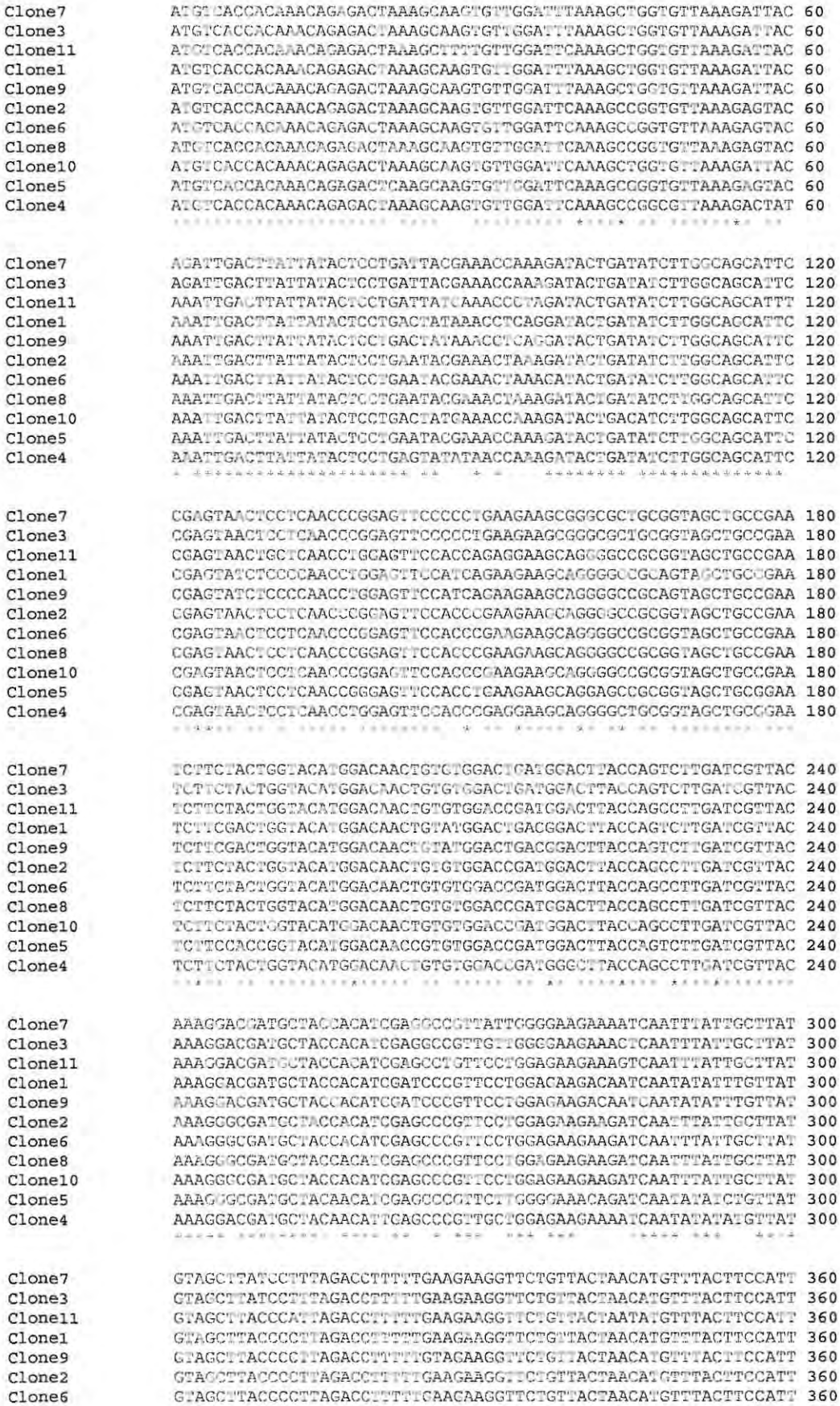
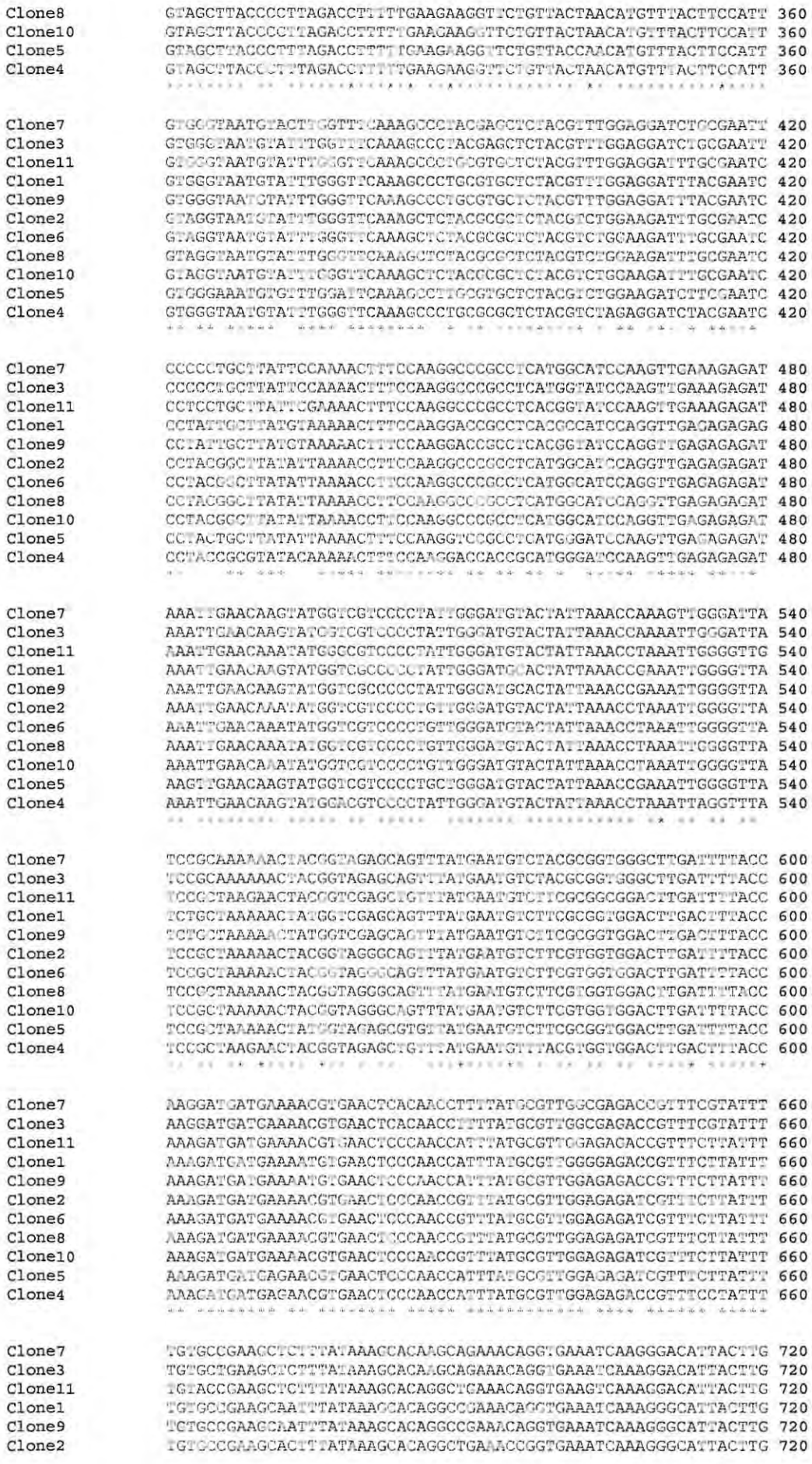
Appendix L: ClustalW 2.0.8 multiple sequence alignment of clones

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Appendix M: Comparison of eleven clone sequences to the GFRR plant sequence database

	Clone number and percent identity										
Plants	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P. auriculata	91.4	92.5	91.6	90.9	89.4	92.5	91.1	92.5	91.8	92.9	**100.0**
E. undulata	92.8	94.4	92.9	92.5	92.5	94.4	92.6	94.4	93.2	94.9	93.0
B. illicifolia	92.7	94.1	90.9	91.8	92.2	94.1	91.0	94.1	92.8	94.6	93.0
P. afra	**96.8**	92.5	90.8	90.9	90.9	92.5	90.6	92.5	**97.1**	92.4	92.9
P. verucosus	91.6	**99.6**	91.9	91.3	92.5	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
C. haematocarpa	91.6	**99.6**	91.9	91.1	92.3	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
M. nemorosa	91.6	**99.6**	91.9	91.1	92.3	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
M. capitata	91.2	92.8	92.4	92.2	91.1	92.8	92.0	92.8	91.5	93.3	92.5
L. ferocissimum	91.2	95.0	91.4	91.8	**93.8**	95.0	91.3	95.0	91.4	94.9	92.4
J. capensis	91.7	92.9	91.8	92.9	91.4	92.9	91.5	92.9	92.0	93.3	92.4
G capitata	91.2	92.8	92.3	92.4	90.9	92.8	92.0	92.8	91.5	93.3	92.3
S. myrtina	91.0	96.0	91.4	91.4	93.0	96.0	91.3	96.0	91.2	95.8	92.1
C. rudis	91.7	92.9	91.4	91.8	90.2	92.9	91.1	92.9	91.8	93.4	92.1
A. tetracantha	90.9	92.6	91.9	92.2	90.7	92.6	91.8	92.6	91.3	93.1	92.1
P. pyracantha	90.5	92.7	91.9	91.8	90.7	92.7	91.6	92.7	90.9	93.2	91.6
E. bothae	91.0	92.5	90.8	92.2	91.4	92.5	90.6	92.5	91.3	92.8	91.6
P. crassicladus	90.2	91.9	**99.6**	89.8	90.2	91.9	**99.4**	91.9	90.4	92.0	91.5
G. robusta	90.7	93.3	90.8	93.0	91.0	93.3	90.8	93.3	90.8	93.2	91.4
P. africanus	90.0	91.4	99.4	89.8	90.2	91.4	98.8	91.4	90.3	91.5	91.4
C. bispinosa	90.2	97.6	90.3	90.0	91.2	97.6	90.1	97.6	90.3	96.9	90.8
O. mucrunata	90.9	92.0	90.0	98.9	90.8	92.0	89.8	92.0	91.1	92.1	90.8
R. pterota	90.0	91.4	89.5	**99.1**	90.2	91.4	89.3	91.4	90.3	91.4	90.5
P. sauveolens	87.7	89.5	97.9	88.1	87.7	89.5	96.9	89.5	87.9	89.6	90.0

Appendix N: Comparison of the *rbc*L FLX generated sequences from black rhinoceros dung samples against the GFRR *rbc*L plant sequence database

Table N1: Summer dung sample

No. of Sequences	Family of match	Genus	Species	% Match
Twenty-seven	**Apocynaceae**	*Carissa*	*bispinosa*	98.8
One	**Ebenaceae**	*Euclea*	*undulata*	96.0
Three	**Portulacaceae**	*Portulacaria*	*afra*	96.3 to 96.6

Table N2: Autumn dung sample

No. of sequences	Family of match	Genus	Species	% Match
Twenty-five	**Apocynaceae**	*Carissa*	*bispinosa*	94.7 to 98.4
Five	**Asteraceae**	*Brachylaena*	*ilicifolia*	91.0 to 95.8
Five	**Celastraceae**	*Gymnosporia, Puttterlickia*	*capitata pyracantha*	80.1 to 98.9
One	**Euphorbiaceae**	*Jatropha*	*capensis*	96
Nine	**Portulacaceae**	*Portulacaria*	*afra*	92.8 to 98.4
Two	**Solanaceae**	*Lycium*	*feroscissimum*	93.9

Table N3: Winter dung sample

No. of sequences	Family of match	Genus	Species	% Match
Thirty-four	**Apocynaceae**	*Carissa*	*bispinosa*	99.2 to 98.4
One	**Anacardiaceae**	*Rhus*	*pterota*	95.4 to 95.9
Eleven	**Portulacaceae**	*Portulacaria*	*afra*	95.5 to 98.8
Two	**Rhamnaceae**	*Scutia*	*myrtina*	92.7 to 95.9

Table N4: Spring dung sample

No. of sequences	Family of match	Genus	Species	% Match
One	**Asteraceae**	*Brachylaena*	*ilicifolia*	96.2
Eleven	**Apocynaceae**	*Carissa*	*bispinosa*	91.2 to 96.9
Five	**Ebenaceae**	*Euclea*	*undulata*	95.0

Appendix O: Reagents for antioxidant assays

Appendix O1: Acetate buffer: 300 mM, pH 3.6 in 1 litre

3.1 g sodium acetate.$3H_2O$

16 ml glacial acetic acid

ddH_2O was added to a litre and stored at 4°C

Appendix O2: PBS, pH 7.4 in 1 litre

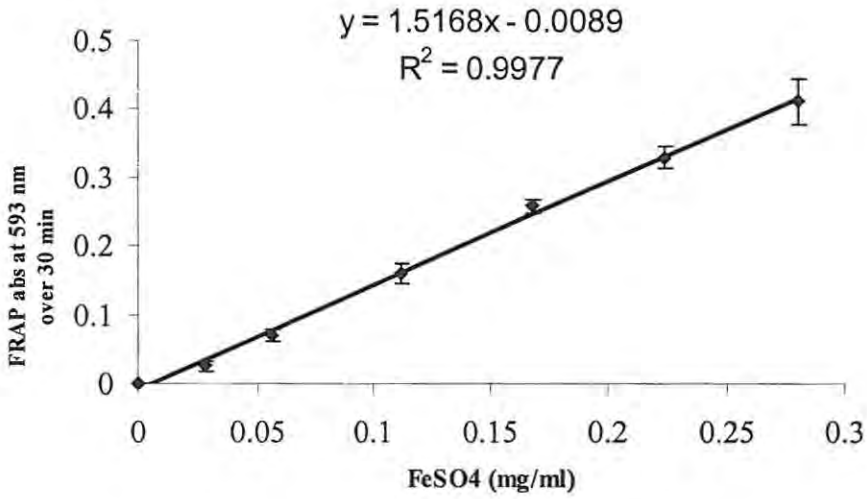
137 mM NaCl

2.7 mM KCl

10 mM $NaHPO_4$

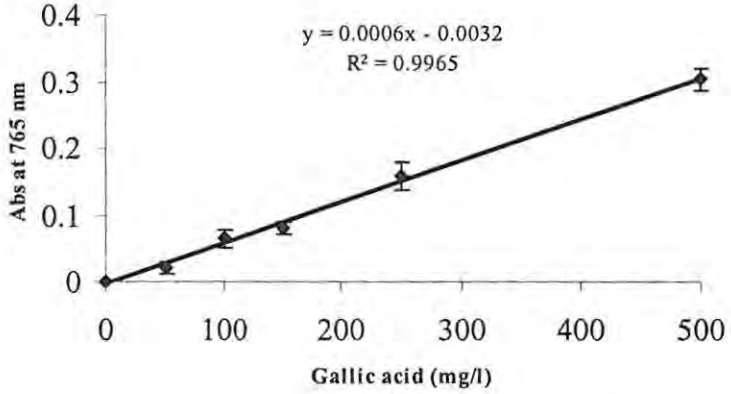
2 mM KH_2PO_4

Appendix P: Ferrous sulfate calibration curve



Concentration response curve for ferrous sulfate standard with the absorbance measured at 593 nm. The experiment was run in triplicate ± SD.

Appendix Q: Gallic acid calibration curve



Concentration response curve for gallic acid standard with the absorbance measured at 765 nm. All data are shown as means ± SD. The experiment was run in triplicate.

Appendix R: Total recovery of plant extracts

Phenolic compounds were extracted from twenty-five different plants representing fourteen families, twenty-one genus and twenty-four species for using 80% methanol

Plant family	Plant species	mg dry weight recovered/ml
Euphorbiaceae	*Jatropha capensis*	246.67
Ebenaceae	*Euclea undulata*	226.62
Celastraceae	*Maytenus heterophylla*	188.33
Boraginaceae	*Ehretia rigida*	180.00
Euphorbiaceae	*Euphorbia bothae*	176.67
Asteraceae	*Brachylaena ilicifolia*	176.67
Asparagaceae	*Protasparagus suaveolens*	170.00
Apocynaceae	*Carissa haematocarpa*	149.50
Portulacaceae	*Portulacaria afra*	146.67
Fabaceae	*Schortia afra*	137.58
Solanaceae	*Lycium ferocissimum*	134.30
Celastraceae	*Maytenus capitata*	133.33
Asparagaceae	*Protasparagus crassicladus*	133.33
Bignoniaceae	*Rhygozum obovatum*	133.33
Capparaceae	*Capparis sepiaria*	133.33
Asparagaceae	*Protasparagus africanus*	133.33
Salvadoraceae	*Azima tetracantha*	133.33
Apocynaceae	*Carissa bispinosa*	130.33
Celastraceae	*Putterlickia pyracantha*	125.00
Anacardiaceae	*Ozoroa mucrunata*	103.33
Anacardiaceae	*Rhus pterota*	100.00
Asteraceae	*Tarchonanthus camphoratus*	97.33
Euphorbiaceae	*Phyllanthus verrucosus*	63.33
Plumbaginaceae	*Plumbago auriculata*	56.67
Tiliaceae	*Grewia robusta*	50.67

Values are the total amount of dry plant material recovered/amount of the initial volume used for extraction. Working solutions of 1 mg/ml of each plant extract were prepared and analyzed for antioxidant activities and total phenolic content.