Chapter 6

Antioxidant activity of *Pteleopsis myrtifolia* leaf extracts

Abstract

Recently there has been increasing interest in free radicals in biological systems and their implied role as causative agents in the aetiology of a variety of pathological physiologies. Antioxidants' potential in preventing damage associated with free radicals and their implied role in disease has encouraged the search for compounds with potent antioxidant activity. This research investigated the antioxidant activity of *Pteleopsis myrtifolia* leaf extracts. The radical scavenger capacities of acetone, chloroform, ethanol, hot water, cold water and methanol leaf extracts were established by using a 340 ATC ELISA plate reader to measure the disappearance of the purple colour of 1,2-diphenyl-2-picrylhydrazyl (DPPH) at 515 nm. The radical scavenger activity was expressed as the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}). Compared to black tea, where one gram of dry weight, had a vitamin C equivalent of 0.126 mg, the cold water extract of *P. myrtifolia* leaves, had a vitamin C equivalent of 0.34 mg, more than twice that of black tea. Further, the vitamin C equivalent of the methanol and hot water extracts were 0.20 and 0.147 mg/g respectively. All *P. myrtifolia* leaf extracts gave positive scavenging capacity (antioxidant) with DPPH. The cold water, methanol and hot water's free radical scavenging or antioxidant activity were more than that of black tea.

6.1 Introduction

6.1.1 Antioxidants protect against oxidative damage

Human consumption of antioxidants has many health benefits, including the prevention of oxidative damage associated with free radical damage and their contribution to disease such as

cancer, the aetiology of aging, coronary heart disease, ischemia-reperfusion injury (Yang *et al*., 2001), multiple sclerosis, Parkinson's disease, senile dementia, autoimmune disorders, and asbestosis (Ng *et al*., 2000). Their protective role has prompted investigators to search for compounds with potent antioxidant activity. Oxygen, vital for the survival of the human species, is present in the atmosphere as a stable triplet biradical ($^3O_2$), in the ground state. Once inside the human body, it can be transformed by a four-electron reduction process to water, producing a super oxide radical (O_2-.), a hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2) as the reactive intermediates. Singlet oxygen ($^1O_2$) is formed from the excited state of various sensitizers (such as chlorophyll, acridine and other pigments) and primarily targets and damages cellular and extra cellular components, proteins, enzymes, lipids, DNA and RNA. It can also oxidize unsaturated fatty acid components of cell membranes (Ramarathnam *et al*., 1995). Free radicals can be described as unstable molecules with unpaired electrons that float freely through the body, seeking out completely healthy cells from with they can 'steal' electrons to rebalance themselves. This can cause a chain reaction in body cells and if left unchecked, can cause cellular damage. These unstable molecules are formed naturally in the body, through normal metabolic processes like oxidation, or they can be generated by car fumes, smoking, factory emissions, alcohol, pesticides, solvents and even from illness or infection. Antioxidant nutrients have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. Most plants have protective biochemical functions of naturally occurring antioxidants in the cells. Many secondary compounds and enzymes of higher plants have been demonstrated with *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Larson, 1988). Naturally occurring antioxidants in plant cells are:

i) enzymatic and peptide defence mechanisms (catalases, peroxidases, superoxide dismutases, glutathione and other proteins),

ii) phenolic defence compounds (vitamin E, flavonoids, phenolic acids and other phenols),

iii) nitrogen compounds (alkaloids, amino acids and amines),

iv) cartenoids, chlorophyll derivatives,

v) other compounds (vitamin C, ketones) and;

vi) synergism between more than one type of compound (for example: phenolic compounds and proteins) (Larson, 1988).

The required compounds would have potent antioxidant activity and low cytotoxicity. Antioxidants present in consumable fruits, vegetables and beverages have received considerable attention as cancer chemopreventative agents (Muktar *et al*., 1994). A study about dietary constituents as potential cancer chemoprotective agents found that resveratrol, sesamol, sesame oil and sunflower oil all had strong anti-tumour promoting effects in both *in vitro* and *in vivo* (mouse skin) models induced by a potent carcinogen. Only sesamol and resveratrol showed profound free radical scavenging activity in 1,2-diphenyl-2-picrylhydrazyl (DPPH) bioassays. The anti-oxidant capabilities of these compounds could not solely explain the observed anticancer characteristics (Kapadia *et al*., 2002). Studies on tea, the most popularly consumed beverage aside from water (associated with decreased risk of various proliferative diseases such as cancer and arteriosclerosis in humans), provided evidence that green tea catechins, in addition to their antioxidative properties, also effect the molecular mechanisms involved in angiogenesis, extracellular matrix degradation, regulation of cell death and multidrug resistance (Demeule *et al*., 2002). Apostolides (1995) found that food-borne carcinogens (produced during food preservation and preparation) were inhibited by tea polyphenols. Research (cell culture and animal models) found that tea's anti-carcinogenic activity was mediated through a range of mechanisms including antioxidant activity, enzyme modulation, gene expression, apoptosis, up regulation of gap junction communication and P-glycoprotein activation (Duthie *et al.*, 2000). A single cup of black or green tea induces a significant rise in the plasma antioxidant activity *in vivo* (Leenen *et al*., 2000).

The use of plant extracts to combat infections or illnesses may be explained by the presence of antimicrobial compounds, but there is also another explanation. If plant extracts contain antioxidant activity, it may clear-up free radicals from the body, and boost the immune system to handle the infection or illness effectively. In the previous chapters 3 and 4, it was shown that *P. myrtifolia* extracts have antibacterial activity.

The aim of research described in this chapter, was to investigate antioxidant activity in extracts from *Pteleopsis myrtifolia* leaves. Free radical scavenging activity was detected with stable DPPH and colour formation (Pezzuto *et al*., 1999).

6.2 Material and methods

6.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

6.2.2 Preparation of plant extracts

Because antioxidant compounds are frequently highly polar compounds, four polar: ethanol, methanol, cold water, and boiling water, as well as an extractant of intermediate polarity: chloroform, were selected. The extraction method described in 2.2.2 of Chapter 2 was used. Yield was determined and the dried extracts (from the extractants were redissolved in methanol. For the DPPH antioxidant assays and the dot-blot DPPH staining procedures, a final concentration of 10 mg/ml of each extractant was prepared by redissolving the dried extract in acetone. For the spectrophotometric assays, each extract was redissolved in methanol. A stock concentration of 1000 mg/ml was prepared from which dilutions were made. The prepared extracts were stored in a tightly sealed dark glass containers at 5 ºC. The extracts were prepared the day previous to the DPPH colour measurements.

6.2.3 DPPH qualitative antioxidant assay

10 µl aliquots of each of the cold water, hot water, ethanol and methanol extracts (10 mg/ml) were applied to Merck Silica gel F_{254} plates. The plates were developed with the chloroform: ethyl acetate: formic acid (5:4:1) (CEF) and ethyl acetate: methanol: water (40:5:4.4) (EMW) eluent systems and sprayed with a 0.4 mM DPPH solution in methanol. This technique indicates how many antioxidant compounds are present.

6.2.4 Dot-blot DPPH staining procedure

According to Soler-Rivas *et al*. (2000), the dot-blot test is an easy, fast and reliable way to compare radical scavenging capacity (RSC) of various food products (excluding oils). This assay was used to establish whether different extracts of *P. myrtifolia* leaves (acetone, chloroform, ethanol, hot water, cold water and methanol) had radical scavenging activity. All the extracts were dried and redissolved in acetone. Aliquots of 5 µl (of a 10 mg/ml final concentration) of each extractant were applied on Merck Silica gel F_{254} plates and allowed to dry for a few minutes. Drops of each sample were placed in a row. The sequence was according to decreasing quantity: 20 µg (bottom row), 10 µg (middle row) and 5 µg (top row). The top four spots were applied in their original extractant. The staining of the TLC plates was done according to the method of Takao *et al*. (1994) with modifications. A 0.4 mM DPPH solution in methanol was sprayed on the plates until they were evenly covered.

6.2.5 Spectrophotometric assays

Extracts of *P. myrtifolia* leaves were prepared with the different extractants (cold water, hot water, ethanol, methanol and chloroform). Not all of the chloroform extract could be redissolved. The 1 g/ml concentration was used as a stock concentration for each extractant and series of dilutions were made separately for each extract. Vitamin C was prepared as a 1

mg/ml solution by adding boiling hot water then filtering it. Black tea was prepared separately as 10 mg/ml by placing 1 g of black tea in 70% ethanol for an hour (Du Toit *et al.*, 2001).

A 96-multi-well plate (MWP) was used as an experimental unit to lie out the different concentrations in triplicate. 180 µl of AR-grade 100% methanol was placed in each well and 20 µl of each sample to be tested was added. 50 µl of a 90 µM solution of DPPH in methanol was added to each well. The plate was covered with aluminium foil and left to stand at room temperature for 1 h before spectrophotometer readings were made.

6.2.6 Spectrophotometer readings

The radical scavenging capacities of the samples were established by using a 340 ATC ELISA plate reader to measure the disappearance of purple colour of DPPH at 515 nm. The radical scavenging activity was expressed as the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Du Toit *et al.*, 2001). The EC_{50} values of the standards and samples were determined and plotted as the percentage disappearance of DPPH as a function of the sample concentration in µg/ml for the standard and mg/ml for the samples.

6.2.6.1 Calculation as a percentage:

The EC_{50} is the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%. The results are expressed as the mg vitamin C equivalents/ g dry weight and are calculated as follows:

EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = X mg vitamin C equivalents/ g dry weight

Zero mg/ml were taken as 100%.

6.3 Results and discussion

6.3.1 DPPH antioxidant assay

Where the Merck silica gel F_{254} TLC plates were sprayed with a DPPH solution in methanol, the regions where substances with antioxidant capacity occurred stained yellow and the remainder of the plate stained purple. Figure 6.1 shows TLC plates of the fractions of liquid-liquid separation cold water, hot water, ethanol and methanol extracts of *P. myrtifolia* leaves developed with the CEF and EMW eluent systems and sprayed with a 0.4 mM DPPH solution in methanol.

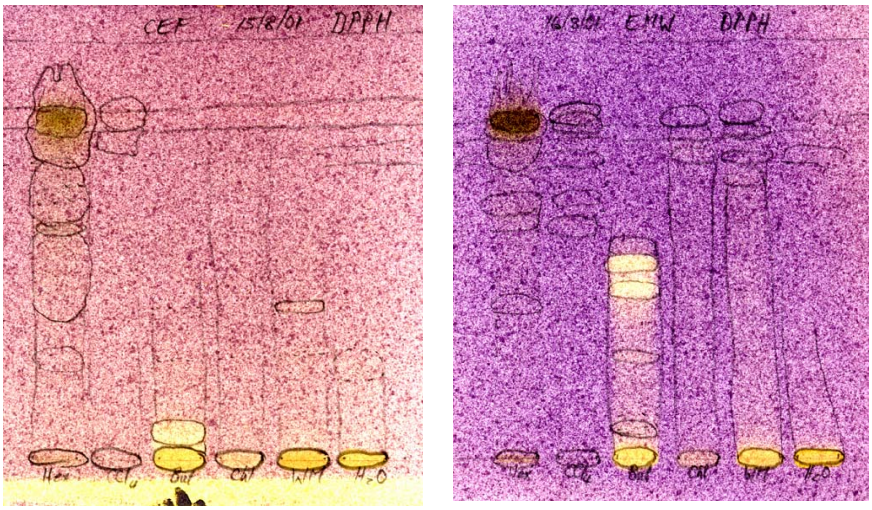


Figure 6.1. Chromatograms of thin layer chromatography plates with fractions from liquid-liquid separation (Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35 % water in methanol, H_2O = water) developed with the CEF (left) and EMW (right) eluent systems and sprayed with a 0.4 mM DPPH solution in methanol. (CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4), DPPH = 1,2-diphenyl-2-picrylhydrazyl).

6.3.2 Dot-blot DPPH staining procedure

The results of the dot-blot assay showed coloured spots where the aliquots of different extracts were placed in rows. The purple area on the plate indicates no free radical scavenging (antioxidant) activity and the yellow areas indicate free radical scavenger or antioxidant activity.

The more intense the yellow colour, the greater the antioxidant activity is (Figure 6.2). The yellow colour can be masked by the green of chlorophyll.

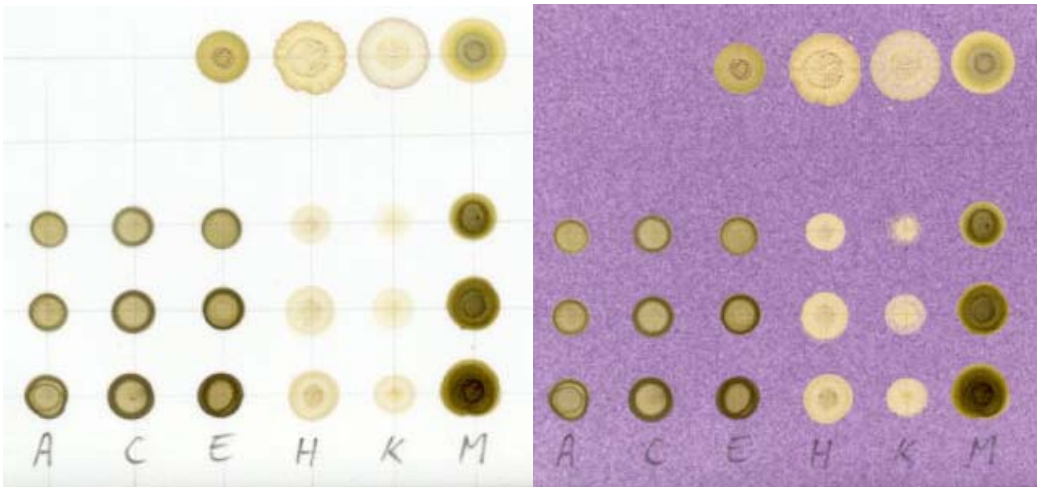


Figure 6.2. Scan of a dot-blot test of a thin layer chromatography (TLC) plate sprayed (right) with a 0.4 mM solution of 1,2-diphenyl-2-picrylhydrazyl in methanol after extracts A, C, E, H, K, and M was applied (A = acetone extract, C = chloroform extract, E = ethanol extract, H = hot water extract, K = cold water extract and M = methanol extract). The dot blots were 20 μg (bottom row), 10 μg (middle row) and 5 μg (top row). The top four spots were dots applied in their original extractant as a 20 μg quantity. Left TLC plate not sprayed yet.

6.3.3 Spectrophotometer readings

The different extract's colour reactions with DPPH in the MWPs were measured by a multiwell plate reader (Figures 6.3 – 6.5).

Vitamin C

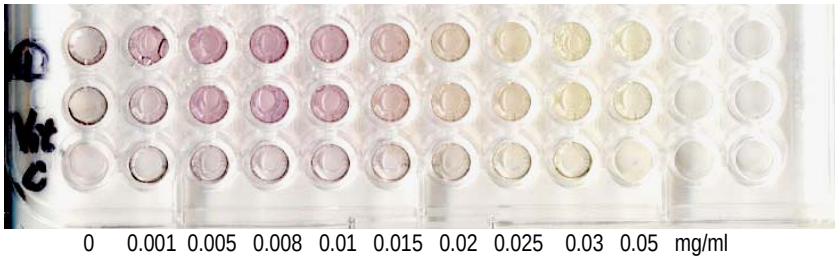


Figure 6.3. Part of a 96-Multiwell plate, showing the gradual colour change with the vitamin C at different concentrations, one hour after addition of 1,2-diphenyl-2-picrylhydrazyl (DPPH).

Ethanol extract (E)

Cold water extract (K)

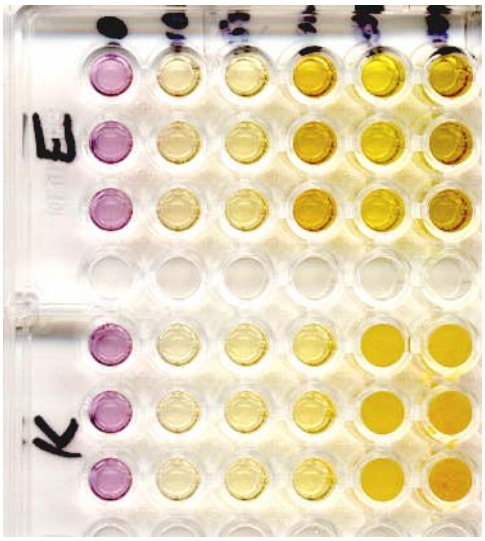


Figure 6.4. Part of a 96-Multiwell plate, showing the colour reaction after addition of 1,2-diphenyl-2-picrylhydrazyl (DPPH) to the initial concentrations (< 50 mg/ml) of cold water and ethanol extracts of *P. myrtifolia* leaves.

Colour formation with DPPH is indicative of a balance between antioxidants and free radicals. Yellow is indicative of antioxidants in excess (and that the concentration of the plant extracts is too high, like in the right hand side of Figure 6.4) and pink of free radicals in excess (and that the concentration of the plant extracts is too low). Therefore a concentration range is sought where the yellow colour just disappears or becomes translucent before pink appears.

Chlorofrom Extract (C)

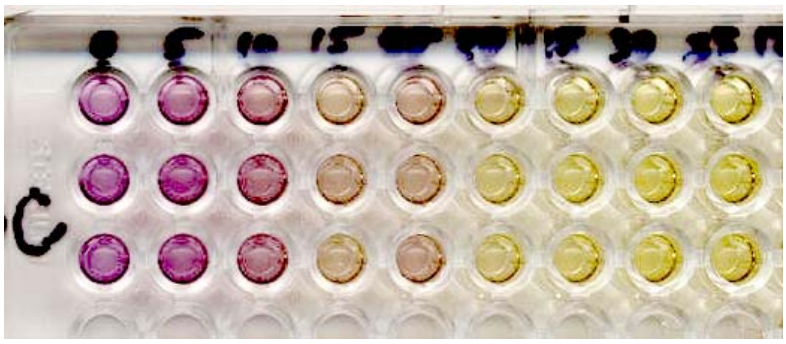
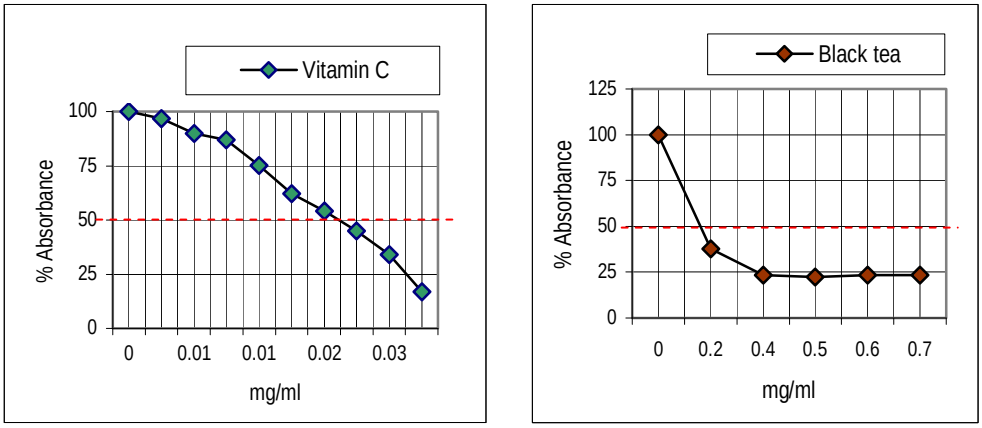


Figure 6.5. Part of a 96-Multiwell plate, showing the colour formation after 1,2-diphenyl-2-picrylhydrazyl (DPPH) was added to the chloroform extract of *P. myrtifolia* leaves.

The colour reaction shows a gradual change from yellow to pink and indicates that the optimum concentration range has been reached.

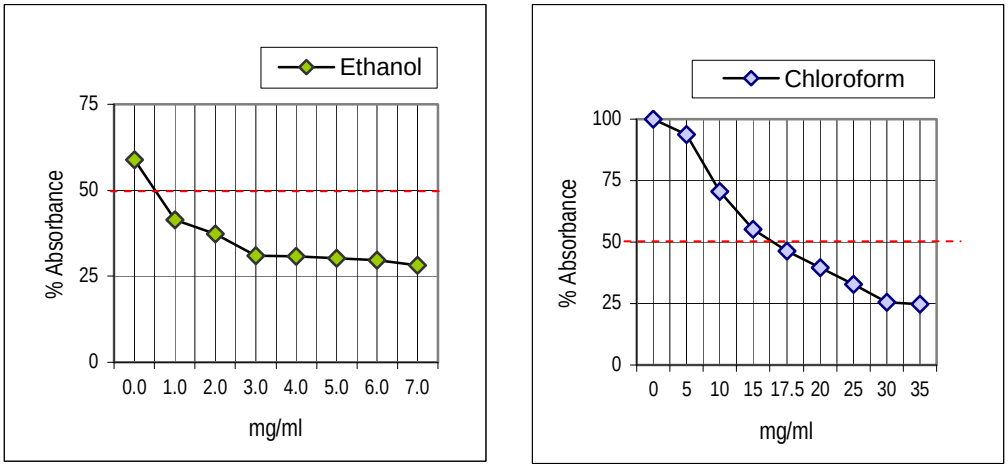
Results of the different extracts are shown in Figures 6.4 and 6.5 and the results that were of the same magnitude were combined and are shown in Figure 6.6. Custom error bars (calculated from three repetitions) are present on all graphs.

The % absorbance of all the graphs (Figures 6.6-6.8) decreased as the amount of extract or Vitamin C increased.



(a) (b)

Figure 6.6. Graphs of (a) vitamin C and (b) black tea plotted against absorbance as measured spectrophotometrically by the colour of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), which gives an indication of free radical scavenger or antioxidant activity.



(c) (d)

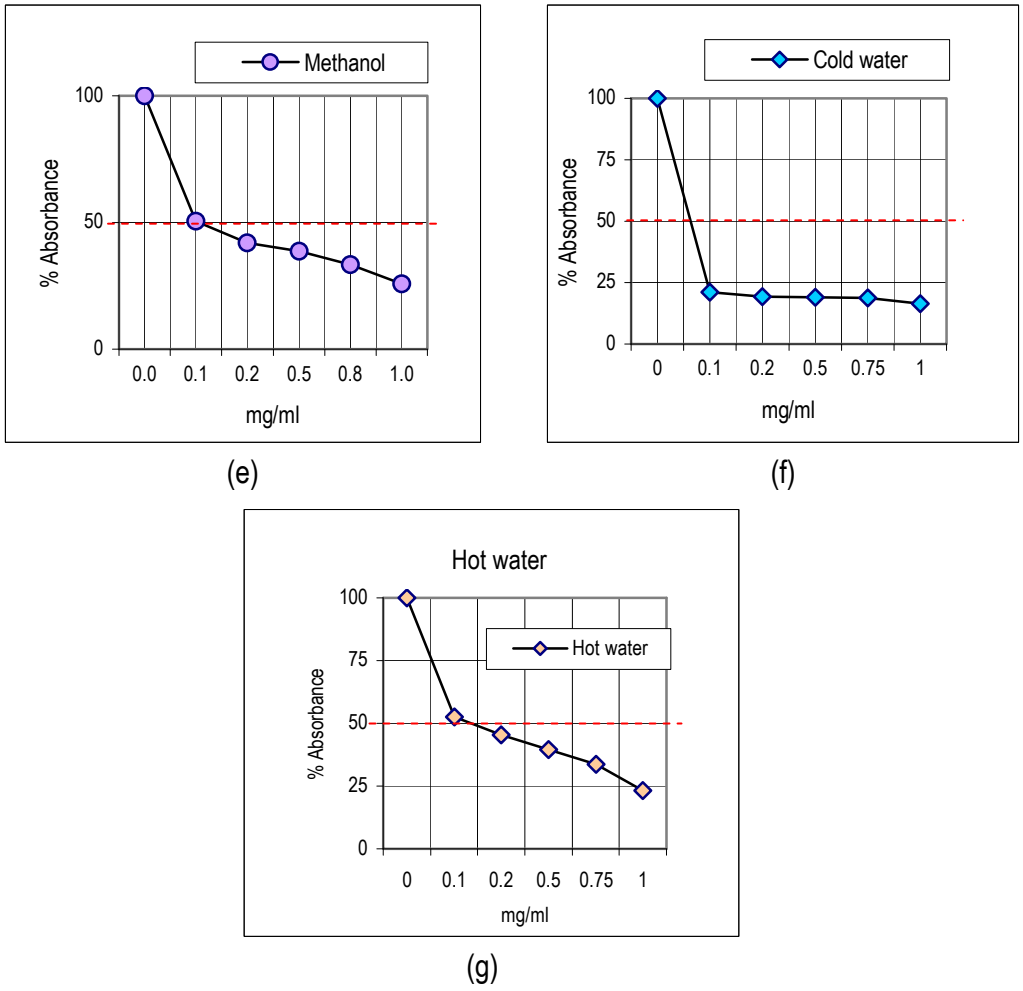


Figure 6.7. Graphs of different *Pteleopsis myrtifolia* leaf extracts (c) ethanol (d) chloroform (e) methanol (f) cold water and (g) hot water plotted against absorbance as measured spectrophotometrically by the colour of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), which indicates free radical scavenger or antioxidant activity.

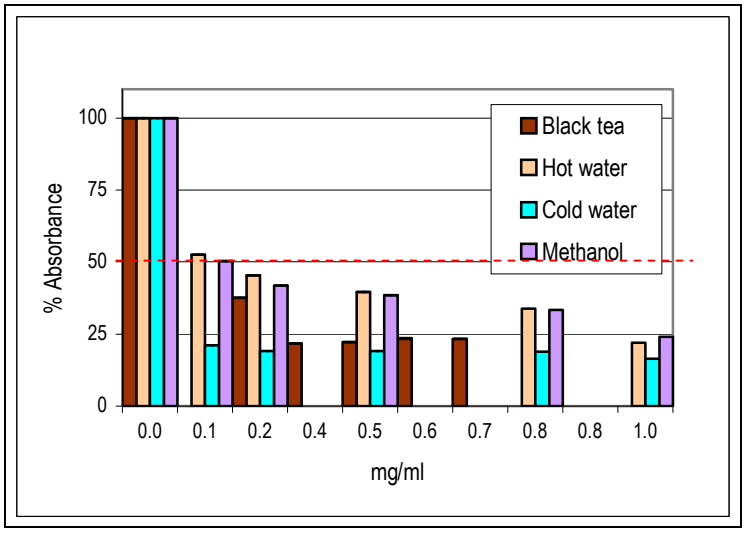


Figure 6.8. Graph of black tea and *Pteleopsis mytifolia* leaf extracts (hot water, cold water and

methanol) plotted against absorbance as measured spectrophotometrically by the colour formation of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), indicating free radical scavenger or antioxidant activity. Error bars for a 5% confidence interval are present.

Expressing plant extracts' antioxidant activity in mg vitamin C equivalent has the benefits that the antioxidant activity is quantified and different plant extracts are comparable. The EC_{50} values (the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%) were calculated (EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = X mg vitamin C equivalents/ g dry weight) and are listed in Table 6.1.

Table 6.1. EC_{50} values for the different *P. myrtifolia* leaf extracts and black tea (Vitamin C's EC_{50} = 0.22 mg/ml).

Extracts	Mg vitamin C equivalents/ g dry weight (EC_{50} value)
Black tea	0.126
Ethanol	0.0138
Chloroform	0.00135
Methanol	0.20
Cold water	0.34
Hot water	0.147

Standard deviation values (present as error bars on graphs) were all ≤ a 1.3% confidence interval.

6.4 Conclusions

The antioxidant activity of the different extracts differed. Compared to black tea, where one gram of dry weight, had a vitamin C equivalent of 0.126 mg, the cold water extract of *P. myrtifolia* leaves, had a vitamin C equivalent of 0.34 mg, more than twice that of black tea. Further, the vitamin C equivalents of the methanol and hot water extracts were both more than

that of black tea, 0.20 and 0.147 mg/g respectively. All *P. myrtifolia* leaf extracts gave positive scavenging capacity (antioxidant) with DPPH.

With these results, it should be taken into account that the *in vitro* free radical scavenging potential of a substance or extract is related to its chemical properties in the medium tested and doesn't necessary reflect the *in vivo* activity.

6.5 Literature references

Apostolides Z (1995) Health aspects of tea. Proceedings of the first regional tea research seminar, Blantyre, Malawi, Montfort Press, Limbe, Malawi

Demeule M, Michaud-Levessque J, Annabi B, Gingras D, Boivin D, Jodoin J, Lamy S, Bertrand Y, Bliveau R (2002) Green Tea Catechins as Novel Antitumor and Antiangiogenic Compounds. Current Medical Chemistry Anti-Cancer Agents 2: 441-463

Du Toit R, Volsteedt Y, Apostolides Z (2001) Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology 166: 63-69

Duthie GG, Duthie SJ, Kyle JAM (2000) Plant polyphenolics in cancer and heart disease: implications as nutritional antioxidants. Nutrition Research Reviews 13: 79-107

Kapadia GJ, Azuine MA, Tokuda H, Takasaki M, Mukainaka T, Konoshima T, Nishino H (2002) Chemopreventive effect of resveratrol, sesamol, sesame oil and sunflower oil in the Epstein-Barr virus early antigen activation assay and the mouse skin two-stage carcinogenesis. Pharmaceutical Research 45: 499-505

Larson RA (1988) The antioxidants of higher plants. Review article 30. Phytochemistry 27: 967-978

Leenen R, Roodenburg AJ, Tijburg LB, Wiseman SA (2000) A single dose of tea with or without milk increases plasma antioxidant activity in humans. European Journal of Clinical Nutrition 54: 87-92

Muktar H, Katiyar SK, Agarwal R (1994) Green tea and skin-anticarcinogenic effects. Journal of Dermatology 102: 3-7

Ng TB, Lui F, Wang ZT (2000) Antioxidative activity of natural products from plants. Life Sciences 66: 709-723

Pezzuto JM, Song LL, Lee LA, Shamon E, Mata-Greewood M, Jung H-L, Pisha E, Mehta RG, Kinghorn AD (1999) Bioassay Methods Useful for Activity-Guided Isolation of Natural Product Cancer Chemopreventive Agents in Hostettman K, Gupta MP, Marston A (Eds.) Chemistry, Biological and Pharmacological properties of medicinal plants from the Americas. Harwood Academic publishers, Australia, pp. 81-110. ISBN 9057023970

Ramarathnam N, Osawa T, Ochi H, Kawakishi S (1995) The contribution of plant food antioxidants to human health. Trends in Food Science and Technology 6: 75-82

Soler-Rivas C, Espin JC, Wichers HJ (2000) An Easy and Fast Test to Compare Total Free Radical Scavenger Capacity of Foodstuffs. Phytochemical analysis 11: 330-338

Takao T, Kitatani F, Watanabe N, Yagi A, Sakata K (1994) A simple screening method for antioxidants produced by marina bacteria from fish and shellfish. Bioscience, Biotechnology and Biochemistry 58: 1780-1783

Yang B, Kotani A, Arai K, Kusu F (2001) Estimation of the Antioxidant Activities of Flavonoids from their Oxidation Potentials. Analytical Sciences 17: 599-60