# CHAPTER 3

# Antioxidants

## 3.1. Introduction

*Combretum* extracts are used for antimicrobial applications. Very low *in vitro* antimicrobial activities were frequently found in water extracts. Water extracts more polar compound and most antioxidants are polar. Plant extracts containing antioxidant compounds may protect patient indirectly by stimulating the immune system. Therefore, I decided to investigate the presence of antioxidant compounds in *Combretum* and *Terminalia* species.

Oxidation in living organisms is essential for the acquirement of energy in catabolism. However, free radicals produced as a result of this process can result in cell death and tissue damage. Free radicals apparently play a role in aging and in diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1999).

Free radicals are continuously produced by our body's use of oxygen such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides, etc. (Li and Trush, 1994). Normally there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems that scavenge/quench these free radicals preventing them from causing deleterious effects in the body (Nose, 2000). The antioxidant defense systems in the body can only protect the body when the quantity of the free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental condition or infections, it leads to oxidative stress, which may result in tissue injury and subsequent diseases (Finkel and Holbrook, 2000).

Plants (fruits, vegetables, medicinal herbs, etc.) contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng and Wang, 2001 and Cai *et al*., 2003). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen *et al*., 2000 and Sala *et al*., 2002).

Some species of the Combretaceae family, have been found to have antioxidant activities. *Terminalia chebula* extracts have different levels of antioxidant activity for anti-lactoperoxidase (LPO), anti-superoxide radical formation and free radical scavenging activities (Cheng *et al*., 2003).

*Terminalia arjuna* is a large tree distributed throughout India and its bark is used as cardioprotective agent in hypertension and ischaemic heart diseases. The bark powder is reported to exert hypocholesterolaemic and antioxidant effects in humans (Gupta *et al*., 2001). Extracts of both *Terminalia sericea* and *Gunnera perpensa* showed possible scavenging activity in a concentration dependant manner. Water extracts demonstrated higher activity than the methanol extracts (Mabogo, 1990). Several galloyl quinic acid derivates have been isolated from the galls of *Guiera senegalensis* (Bouchet *et al*., 1996) and have shown antioxidant activity (Bouchet *et al*., 1998).

Masoko *et al*., (2005), have reported that six *Terminalia* species tested possess antioxidant activity. But less work has been done on *Combretum* species. Although many synthetic chemicals, such as phenolic compounds are strong radical scavengers, they usually have side effects (Imaida *et al*., 1983). Antioxidant substances obtained from natural sources may be of great interest in the near future.

### *3.1.1. Antioxidant screening*

The most commonly used methods for measuring antioxidant activity are those that involves the generation of a free radical species, which are then neutralized by antioxidant compounds (Arnao *et al*., 2001). Free radicals are the main focus in research related to antioxidants and oxidative stress. They are reactive species (oxidants), generated internally and externally, that can have adverse effects on physiological function. A free radical is defined as an atom or molecule having at least one unpaired electron. Free radicals generally abstract electrons from other molecules, thereby inducing a chain reaction of electron abstraction and radical formation.

In qualitative analysis of antioxidant activity, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screen test for the radical scavenging ability of the compounds present in the different extracts. DPPH is a purple coloured compound that does not dimerize and can hence be prepared in crystalline form. It is a stable free radical and following interaction with antioxidants, they either transfer electrons or hydrogen atoms to it thus neutralizing its free radical character (Naik *et al*., 2003).

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to it will reacts with DPPH, thus bleaching its colour, through reduction from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine as a major product whilst 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes which is shown from **figure 3.1**. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Naik *et al*., 2003).

The main objective was to evaluate the antioxidant activity of various extracts from *Combretum* and *Terminalia* species, and to choose one with the promising antioxidant to do further studies.

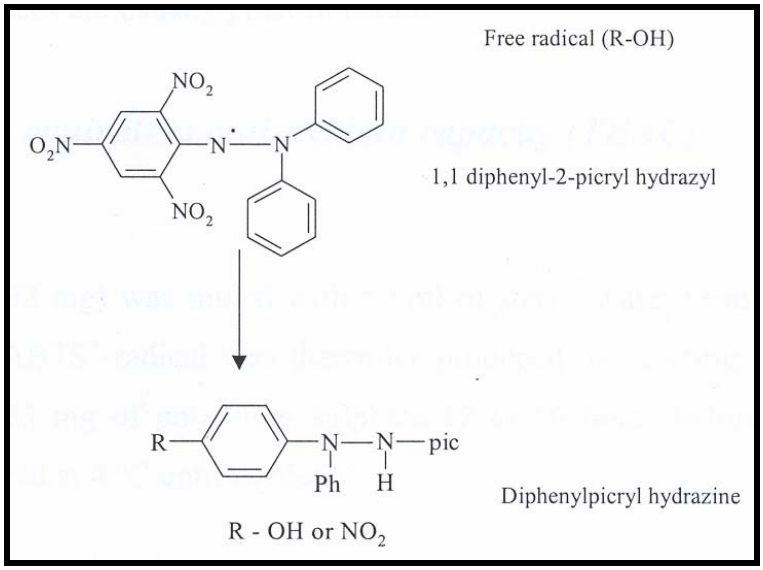



**Figure 3.1**. Reaction of DPPH with hydroxyl groups of free radical (R-OH) to produce 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine and R-NO2, 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine

## 3.2. Materials and Methods

### *3.2.1. TLC-DPPH antioxidant screening*

This method is generally used for the screening of potential antioxidant activity in crude plant extracts. It involves the chromatographic separation of the crude plant extract, after which

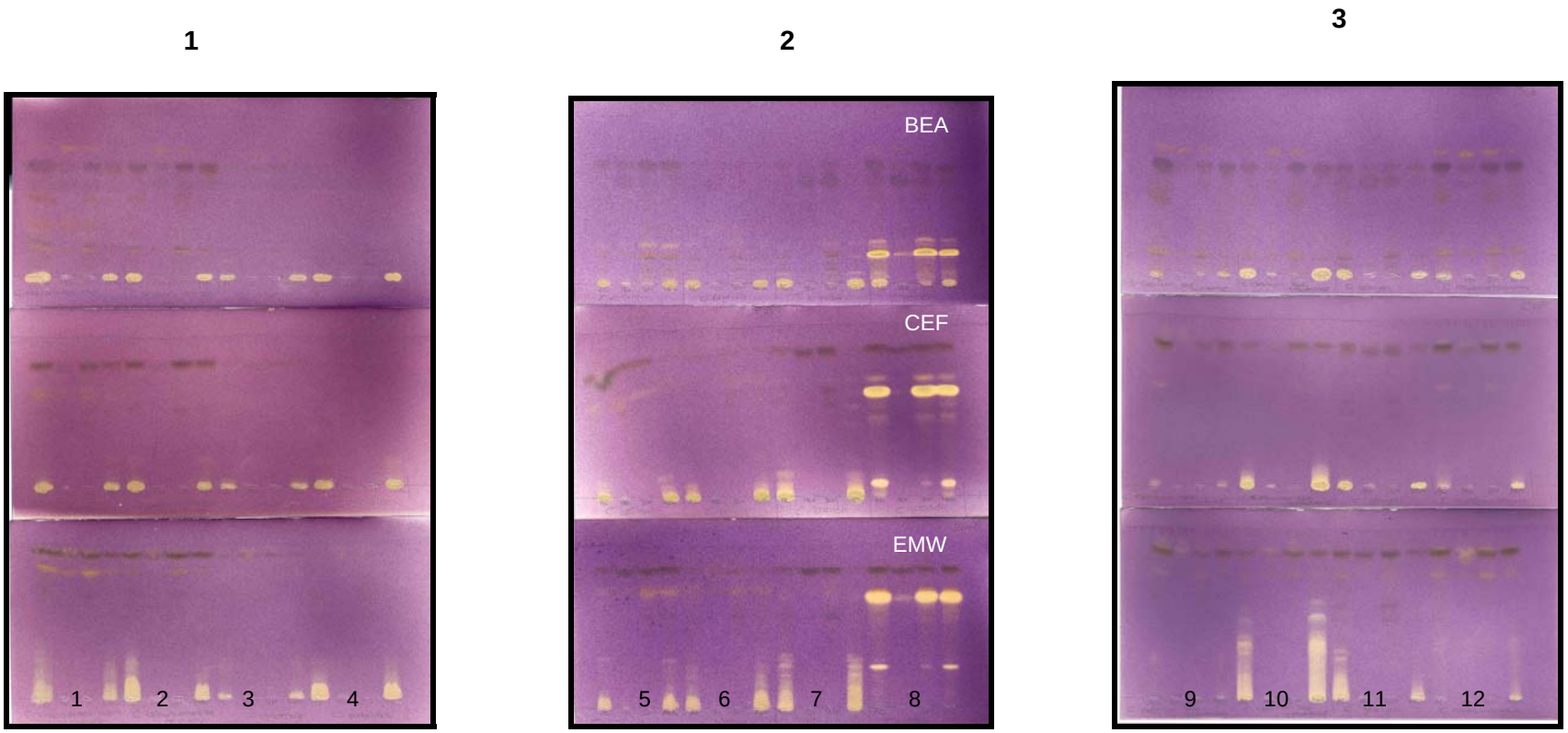
the developed chromatogram is sprayed with a coloured radical solution and the presence of antioxidant compounds is indicated by the disappearance of the radical's colour. Ten microlitres of each extract was loaded as a 1 cm band on the origin of the TLC (Merck, silica gel 60 $F_{254}$) plates. Plates were developed using BEA, CEF and EMW (**Section 2.2.5**). Plates were viewed under UV (254 and 360 nm) light to locate the UV active compounds. To detect antioxidant activity, chromatograms were sprayed with 0.2 % 1.1 diphenyl-2-picryl-hydrazyl (Sigma®)(DPPH) in methanol, as an indicator (Deby and Margotteaux, 1970) until just wet, and dried in the fumehood. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol.

### 3.3. Results and Discussion

TLC-DPPH screening method indicated the presence of antioxidant compounds in some of the extracts tested, with *C. woodii* and *C. hereroense* showing the most prominent antioxidant activity (**Figures 3.2a** to **3.2c**). Visualization of the compound with antioxidant activity enabled the localization and the subsequent identification of the potential active compounds.

Results of chromatograms sprayed with 0.2 % DPPH are presented in **Figures 3.2a** to **3.2c**. The acetone and methanol extracts had antioxidant activity after spraying chromatogram. Hexane and dichloromethane extracts apparently did not have any antioxidant activity in *Terminalia* species but hexane and dichloromethane extracts of *Combretum* showed activity, although most of them were very polar. Most of antioxidant compounds were observed in EMW.

*C. woodii* (**Figure 3.2a(2)**) had very clear antioxidant active compounds from acetone, DCM and methanol extracts. The most prominent compounds were at $R_f$ values 0.20 (BEA), 0.65 (CEF) and 0.73 (EMW). *C. kraussii* also had antioxidant activity especially in EMW. In **Figure 3.2a(3)** only *C. collinum* ssp. *taborense* had antioxidant compounds in EMW from acetone and methanol extracts. The acetone extract of *C. zeyheri* had active compounds with less activity. **Figure 3.2b(4)** had less active compounds calorimetrically determined , but *C. apiculatum* ssp. *apiculatum* showed a number of them and *C. molle* and *C. moggii* thus have some activity.



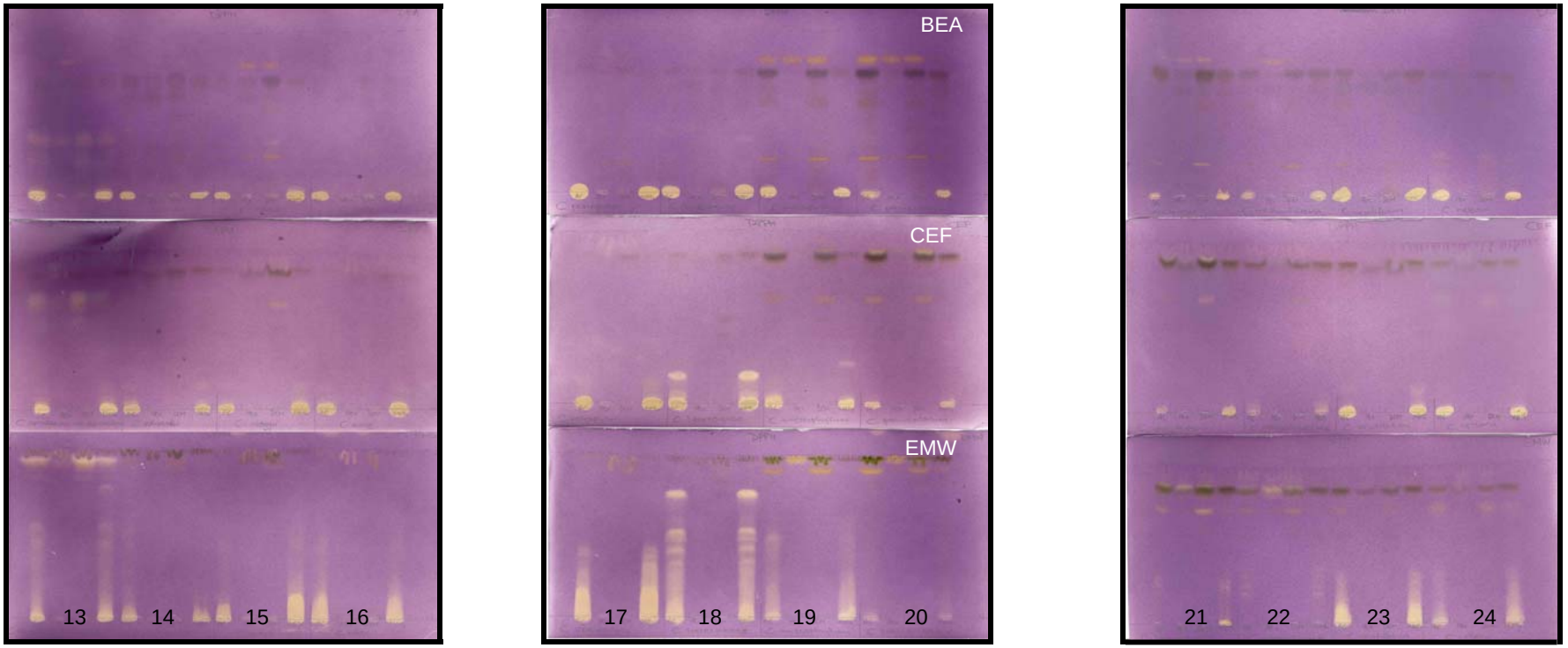

1 *C. celastroide* ssp *celastroides*
*2 C. celastroides* ssp. *orientale*
3 *C. imberbe*
*4 C. padoides*

5 *C. caffrum*
6 *C. erythrophyllum*
7 *C. kraussii*
8 *C. woodii*

9 *C. collinum* ssp. *suluense*
10 *C. collinum* ssp. *taborense*
11 *C. zeyheri*
12 *C. albopunctatum*

**Figure 3.2a**. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.

**4** **5** **6**

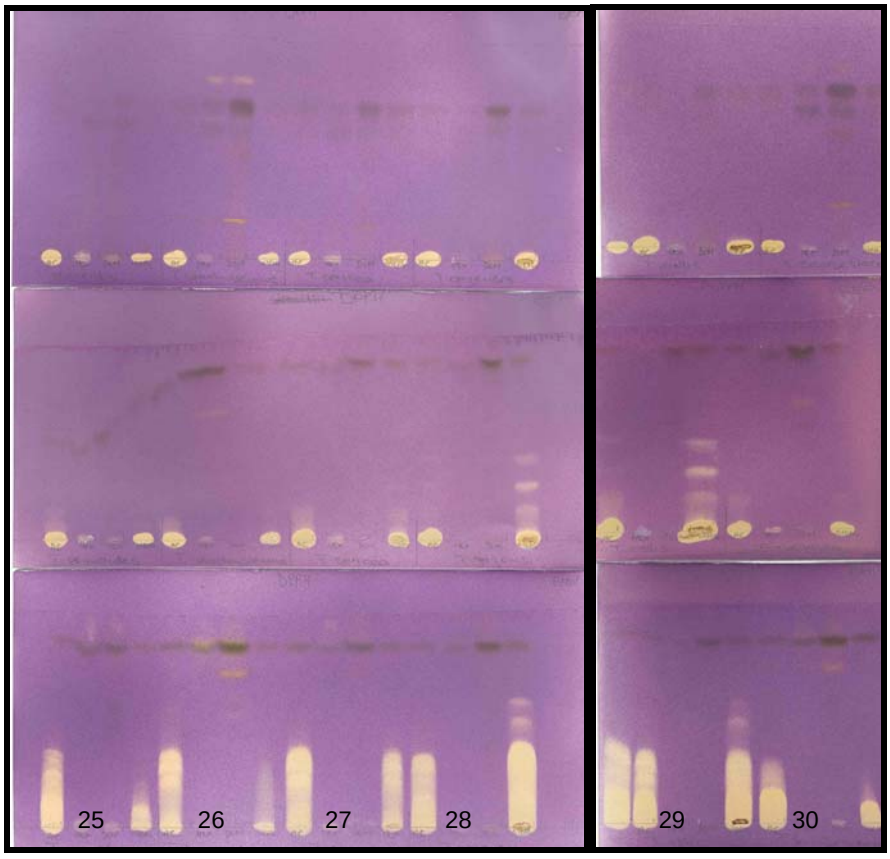



13 *C. apiculatum* ssp. *apiculatum*
14 *C. edwardsii*
15 *C. moggii*
16 *C. molle*

17 *C. petrophilum*
18 *C. hereroense*
19 *C. microphyllum*
20 *C. paniculatum*

21 *C. bracteosum*
22 *C. mossambicense*
23 *C. acutifolium*
24 *C. nelsonii*

**Figure 3.2b**. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.




25 *T. prunioides*
26 *T. brachystemma*
27 *T. sericea*
28 *T. gazensis*
29 *T. mollis*
30 *T. sambesiaca*

**Figure 3.2c**. Chromatograms of *Terminalia* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.

*C. hereroense* (**Figure 3.2b(5)**) also had good number of antioxidant compounds to isolate active compounds from, and these compounds are clearly shown in CEF and EMW systems. *C. petrophilum* and *C. microphyllum* have antioxidant compounds. In **Figure 3.2b(6)** none of the tested *Combretum* species had prominent activity, but *C. acutifolium* had less activity in EMW system.

All *Terminalia* species (**Figures 3.2c**) had activity in the acetone and methanol extracts. *T. gazensis* and *T. mollis* methanol extracts had a number of antioxidant compounds in CEF and EMW. The degree of activity of all the samples tested was determined qualitatively from observation of the yellow colour intensity, which indicate antioxidant activity (**Table 3.1**). Only *C. woodii* and *T. mollis* showed activity in other extracts, other

than acetone and methanol, that is in DCM and hexane, respectively

BEA and CEF solvent systems had fewer active compounds, and active compounds were only observed with EMW as eluent for *Combretum* species (**Table 3.2**) and *Terminalia* species (**Table 3.3**). *C. hereroense* had the highest number of active compounds (16), followed by *C. collinum* ssp. *taborense* (10). Acetone extracts of all tested *Combretum* species had 53 active band, methanol extracts had 55, and DCM extracts had only 3 from *C. woodii* (**Table 3.2**). There are differences in species in same section. In *Metallicum* section *C. collinum* ssp *suluense* did not have antioxidant activity but *C. collinum* ssp *taborense* had 10 active bands, in *Connivetia* section *C. microphyllum* had 6 active bands and *C. paniculatum* had nothing, and in *Poivrea* section *C. acutifolium* had 4 and *C. bracteosum* and *C. mossambicense* had nothing. It appears that the presence of antioxidant compounds does not correlate well with taxonomy based on morphological characters.

Six tested *Terminalia* species had the same number of active compounds in the acetone extracts (24) (**Table 3.3**) extracts, and in methanol (23). *T. mollis* hexane leaf extracts had 4 antioxidant compounds, and it was the only species with activity in the hexane extract. Again species in same sections have different number of active compounds. In *Psidiodes* section, *T. brachystemma* had 6 active compounds and *T. sericea* had 8. In *Platycarpae* section *T. sambesiaca* had 6 active compounds and *T. gazensis* and *T. mollis* had 11 and 14 respectively.

**Table 3.1**. Qualitative DPPH assay on TLC of the 30 plants studied

| **Plant species** | Extractants | | | |
|---|---|---|---|---|
| | Acetone | Hexane | DCM | Methanol |
| ***Combretum* species** | | | | |
| *C. celastroides* ssp. *celastroides* | ++ | - | - | ++ |
| *C. celastroides* ssp. *orientale* | ++ | - | - | ++ |
| *C. imberbe* | ++ | - | - | ++ |
| *C. padoides* | ++ | - | - | ++ |
| *C. caffrum* | ++ | - | - | ++ |
| *C. erythrophyllum* | ++ | - | - | ++ |
| *C. kraussii* | ++ | - | - | ++ |
| *C. woodii* | +++ | - | +++ | +++ |
| *C. collinum* ssp. *suluense* | - | - | - | - |
| *C. collinum* ssp. *taborense* | +++ | - | - | +++ |
| *C. zeyheri* | ++ | - | - | + |
| *C. albopunctactum* | - | - | - | + |
| *C. apiculatum* ssp. *apiculatum* | ++ | - | - | ++ |

| | | | | |
|---|---|---|---|---|
| *C. edwardsii* | ++ | - | - | + |
| *C. moggi* | + | - | - | ++ |
| *C. molle* | ++ | - | - | ++ |
| *C. petrophilum* | ++ | - | - | ++ |
| *C. hereroense* | +++ | - | - | +++ |
| *C. microphyllum* | + | - | - | + |
| *C. paniculatum* | - | - | - | - |
| *C. bracteosum* | - | - | - | - |
| *C. mossambicense* | - | - | - | - |
| *C. acutifolium* | ++ | - | - | ++ |
| *C. nelsonii* | ++ | - | - | ++ |
| | | | | |
| ***Terminalia* species** | | | | |
| *T. prunioides* | +++ | - | - | ++ |
| *T. brachystemma* | +++ | - | - | + |
| *T. sericea* | +++ | - | - | +++ |
| *T. gazensis* | +++ | - | - | +++ |
| *T. mollis* | +++ | +++ | - | +++ |
| *T. sambesiaca* | +++ | - | - | + |

The degree of activity, determined qualitatively from observation of the yellow colour intensity: weak (+), moderate (++), strong (+++) and no activity (-)

**Table 3.2**. Number of antioxidant bands present in all *Combretum* species tested on EMW solvent systems and extractants.

| ***Combretum* species** | **Extractants** | | | | **Total** | **Section** |
|---|---|---|---|---|---|---|
| | **Acetone** | **Hexane** | **DCM** | **Methanol** | | |
| *C. celastroides* ssp. *celastroides* | 3 | | | 3 | **6** | **H** |
| *C. celastroides* ssp. *orientale* | 3 | | | 1 | **4** | **H** |
| *C. imberbe* | 1 | | | 1 | **2** | **H** |
| *C. padoides* | 2 | | | 2 | **4** | **H** |
| *C. caffrum* | 1 | | | 1 | **2** | **A** |
| *C. erythrophyllum* | 1 | | | 2 | **3** | **A** |
| *C. kraussii* | 3 | | | 3 | **6** | **A** |
| *C. woodii* | 3 | | 3 | 3 | **9** | **A** |
| *C. nelsonii* | 3 | | | 3 | **6** | **A** |
| *C. collinum* ssp. *suluense* | 0 | | | 0 | **0** | **M** |
| *C. collinum* ssp. *taborense* | 4 | | | 6 | **10** | **M** |
| *C. zeyheri* | 3 | | | 1 | **4** | **S** |
| *C. albopunctactum* | 0 | | | 1 | **1** | **C** |
| *C. apiculatum* ssp. *apiculatum* | 3 | | | 6 | **9** | **C** |
| *C. edwardsii* | 3 | | | 1 | **4** | **C** |
| *C. moggi* | 2 | | | 2 | **4** | **C** |
| *C. molle* | 2 | | | 2 | **4** | **C** |
| *C. petrophilum* | 3 | | | 4 | **7** | **C** |
| *C. hereroense* | 8 | | | 8 | **16** | **B** |

| | | | | | | |
|---|---|---|---|---|---|---|
| *C. microphyllum* | 3 | | | 3 | **6** | **Co** |
| *C. paniculatum* | 0 | | | 0 | **0** | **Co** |
| *C. bracteosum* | 0 | | | 0 | **0** | **P** |
| *C. mossambicense* | 0 | | | 0 | **0** | **P** |
| *C. acutifolium* | 2 | | | 2 | **4** | **P** |
| TOTAL | **53** | | **3** | **55** | **108** | |

H, *Hypocrateropsis*; A, *Angustimarginata*; M, *Metallicum*; C, *Ciliatipetala*; B, *Breviramea*, Co, *Connivetia*; P, *Poivrea*

**Table 3.3**. Number of antioxidant bands present in all *Terminalia* species tested on EMW solvent systems and extractants.

| ***Terminalia* species** | **Extractants** | | | | **Total** | **Section** |
|---|---|---|---|---|---|---|
| | **Acetone** | **Hexane** | **DCM** | **Methanol** | | |
| *T. prunioides* | 4 | | | 2 | **6** | **A** |
| *T. brachystemma* | 4 | | | 2 | **6** | **Ps** |
| *T. sericea* | 4 | | | 4 | **8** | **Ps** |
| *T. gazensis* | 4 | | | 7 | **11** | **Pl** |
| *T. mollis* | 4 | 4 | | 6 | **14** | **Pl** |
| *T. sambesiaca* | 4 | | | 2 | **6** | **Pl** |
| **TOTAL** | **24** | **4** | | **23** | **51** | |

A, *Abbreviatae*; Ps, *Psidiodes*; Pl, *Platycarpae*

### 3.4. Conclusion

The leaves of Combretaceae family are known for their pharmacological activity and in this chapter it has been shown that many extracts also contain several anti-oxidant compounds. Plants with the best antioxidant effects were *C. woodii*, *C. collinum* ssp. *Taborense, C. hereroense, T. gazensis* and *T. mollis* Methanol and acetone extracted the most antioxidant compounds based on DPPH TLC. I*n vitro* studies coupled with the phytochemical analysis confirm that the extracts possessed potential antioxidant activity. Qualitative DPPH assay on TLC method established was successfully used in this study to systematically assess the total antioxidant capacity of the *Combretum* and *Terminalia* species extracts on a large scale, being simple, fast, reliable, inexpensive, and also very adaptable to both hydrophilic and lipophilic antioxidants systems.