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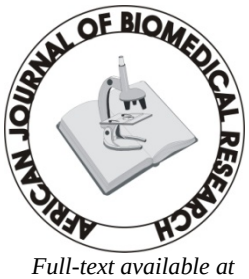

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Full Length Research Article

Free Radical Scavenging Activity of Crude Extracts and 4´-O-Methylepigallocatechin Isolated From Roots of *Cassine Transvaalensis* Burtt-Davy from Botswana

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ABSTRACT

Water and ethanol extracts of roots from *Cassine transvaalensis* Burtt-Davy (celastraceae) were assessed for *in vitro* antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The ethanolic extract exhibited higher free radical scavenging effect than the water extract at all tested concentrations. Above 100µg/ml, the ethanolic extract showed 80% scavenging activity, similar to control antioxidant compounds quercetin, rutin and L-ascorbic acid. The water extract reached a similar level of activity (80%) at 200µg/ml. Between 20-50µg/ml, 4'-O-methyl-epigallocatechin isolated by bioassay directed fractionation exhibited scavenging activity greater than that of either the ethanolic or aqueous crude extract. However, at concentrations above 50µg/ml, the scavenging activity of the ethanolic extract exceeded that of 4'-O-methyl-epigallocatechin.The results suggest that extracts from the roots of *Cassine transvaalensis* have strong antioxidant activity. These findings support the ethnomedical use of this plant to promote good health.
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Key Words; *Cassine transvaalensis; antioxidant; DPPH radicals; 4'-O-methyl-epigallocatechin; epicatechin*

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INTRODUCTION

Cassine transvaalensis Burtt-Davy (vernacular name -monamane) is a small medium-sized tree much branched with rigid, arching stems. It occurs in bushveld, occasionally on termitaria. It is widely distributed along the eastern to South eastern Botswana. The roots are used extensively in traditional medicine to treat backache. In Botswana, a decoction of the root is believed to treat arthritis (Source: traditional healer). The Zulu people in South Africa drink large quantities of the root infusion as a general stomach conditioner (Source: traditional healer). It is also used to prepare an enema to relieve stomach aches and fevers (Coates-Palgrave, 1977). These ethnomedical uses prompted us to investigate the roots extracts for antioxidant activity using the DPPH free radical scavenging assay and to further isolate the principles responsible for the antioxidant activity.

Reactive oxygen species (ROS), including free radicals such as superoxide anion radicals(O^{-}_{2}), hydroxyl radical species ($OH^{\cdot}$), singlet oxygen ($^{1}O_{2}$), and hydrogen peroxide, are active oxygen species that are often generated by biological oxidation reactions of exogenous factors (Crutti, 1991; Halliwell and Gutteridge, 1990). These ROS are known to cause severe damage to biological molecules (Aruma, 1994; Kehrer, 1993). Several phenolic compounds from plants can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Lewis, 1993; Rao, Paliyath & Ormrod, 1996).

Antioxidants are of great importance in terms of reducing oxidative stress that is thought to cause damage to biological molecules (Bektas *et al*, 2005). Several studies have described the antioxidant properties of medicinal plants rich in phenolic compounds (Tsao, 2004; Nijveldt *et al*, 2001). Natural antioxidants such as α-tocopherol and L-ascorbic acid are widely used because they are seen as being safe and causing few adverse effects, but their antioxidants effects are however, lower than those of synthetic antioxidants such as butylated hydroxytoluene (BHT) (Seung Hwa baek *et al*, 2004). Hence, the need exists for safe and economic antioxidants with high activity from natural sources to replace these synthetic chemicals. Medicinal plants represent a constant interest as sources of new antioxidant substances. The large majority of substances isolated from plants with antioxidant activities are flavonoids (Nijveldt *et al*, 2001). Some of the best described flavonoids, are qurcetin, from appleskin (Tagliaferro *et al*, 2002); myricetin, from red wine (Tagliaferro *et al* 2002); (-)epicatechin, from tea (Lopez *et al*, 2001); rutin, from red wine (Hara *et al*, 1995); luteolin, from red pepper (Hertog *et al*, 1992). These compounds are able to scavenge free radicals and thus inhibit oxidative mechanisms that lead to oxidative stress. Oxidative stress can lead to inactivation of cellular components and can have serious effects on the cells, probably leading to ageing as well as several diseases (Kerry *et al., 2003*).

The root bark of *Cassine transvaalensis* contains a pentogynoid, (+)-11,11-dimethyl-1,3,8,10tetrahydroxy-9-methoxypeltogynan and pentacyclic triterpenes; canaphylol, canophyllal, 6-beta-hydroxylup-20(30-en-3-one (Drewes *et al*., 1991).

MATERIALS AND METHODS

Plant materials and extraction

The plant was collected from Goo-Mosweu, in Tswapong North, Botswana. The botanical identification of the collected plant was done by Dr Bruce Hegreaves; (PhD plant taxonomy) and Queen Turner of the National Herbarium and Gallery- Gaborone, Botswana where voucher specimen (DMT 61) has been deposited.

Powdered root material was extracted in water or ethanol (2.5l) using soxhlet apparatus for 48hours. The resultant crude ethanol extract was evaporated to dryness using rota vapour whilst the aqueous extract was concentrated using a freeze drier. The dried crude extracts were then stored in the fridge until ready for use.

Chemicals

DPPH (Sigma), positive controls: [Quercetin (Fluka), Rutin (Sigma), Chrysin (Aldrich), (-)epicatechin (Fluka), L-ascorbic acid]; *Cassine transvaalensis* water and ethanol extracts, 4'-O-methyl-epigallocatechin and ethanol (AR).

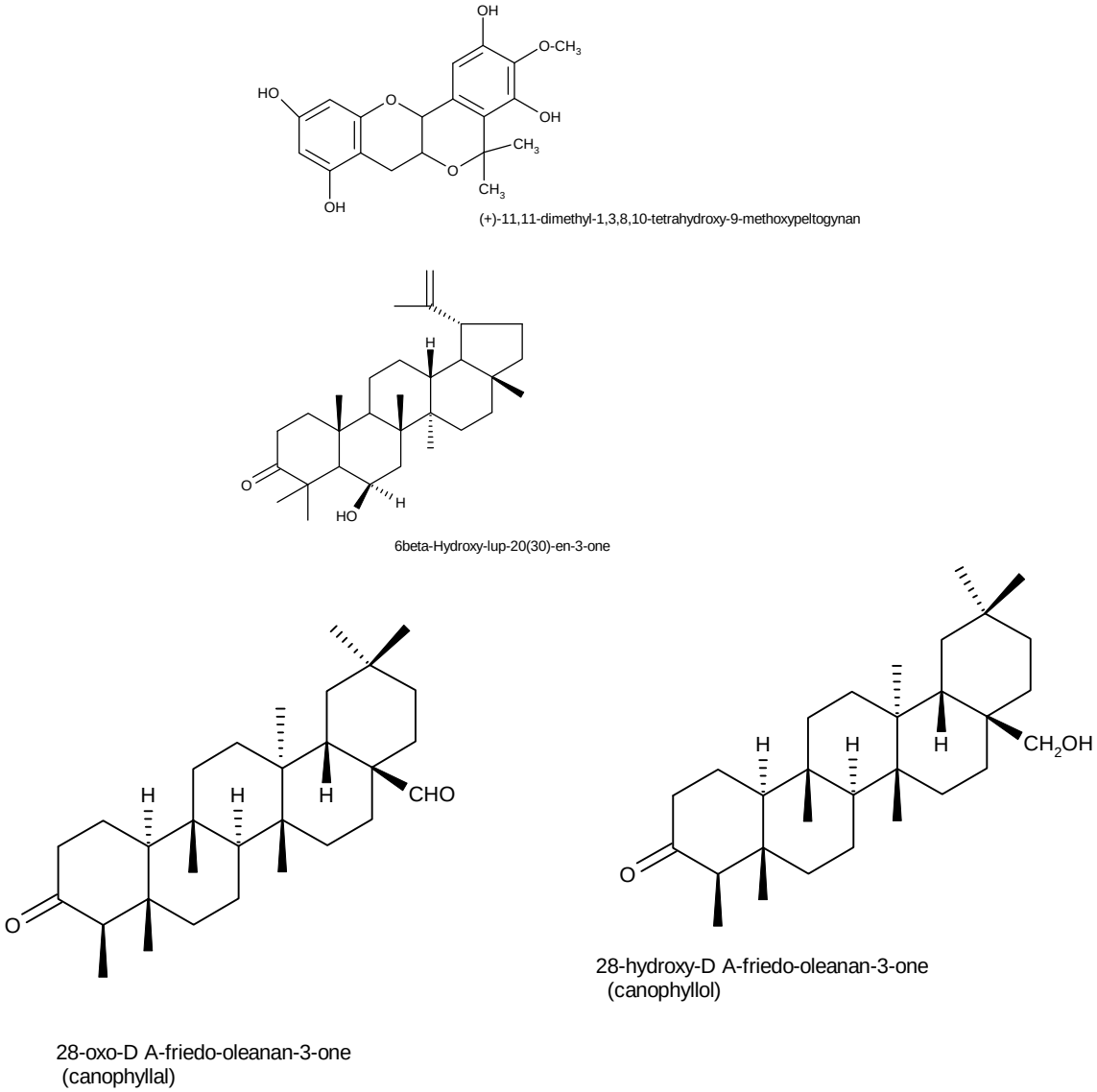


Figure 1:
Previously isolated compounds from root bark of *Cassine transvaalensis* Burtt-Davy

Plant material

The plant was authenticated by comparison with herbarium sample at the Botswana National Herbarium and Gallery, Gaborone, Botswana, where voucher specimen has been deposited.

Thin layer chromatography (TLC)

The TLC plates used were analytical TLC (Merck Si-gel 60 F_{254nm} 0.25mm thick). A TLC plate spotted with 80-100µg of sample (1mg/ml) in methanol, was developed using a mixture of Petroleum spirit/ethylacetate/methanol/water (30:70:30:10) (fig. 1-2). After drying, the plate was sprayed with a 0.2% solution of DPPH (Sigma) in methanol and heated at 100ºC for 5 minutes. Active compounds appeared as yellow spots against a purple background. Plant extracts that showed activity were further subjected to fractionation to identify the compounds responsible for the antioxidant activity.

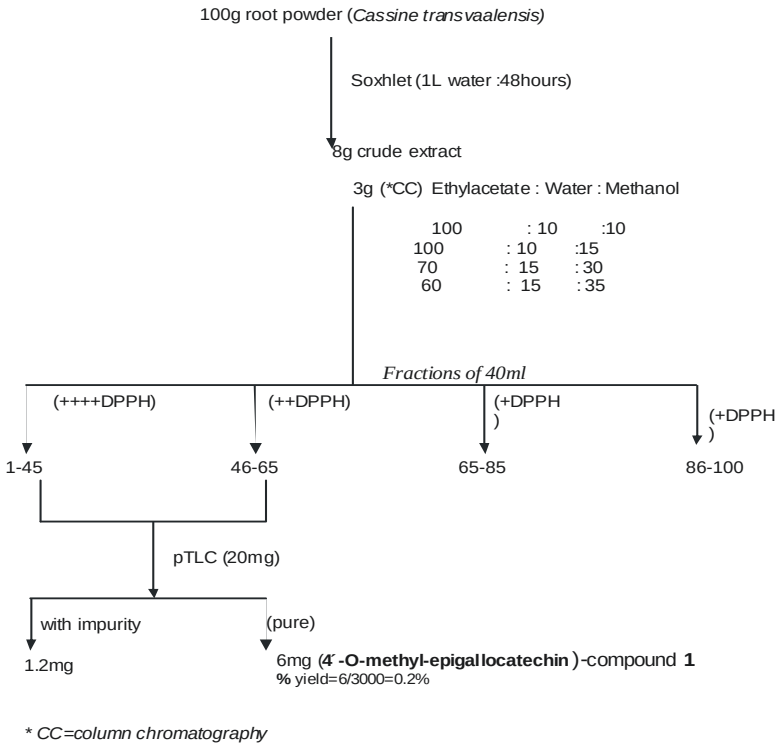


Figure 1:
Scheme of isolation for 4´-O-methyl-epigallocatechin (compound 1) from water extract of *Cassine transvaalensis* Burtt-Davy. *Key: (+++) denotes strong antioxidant activity; (++) denotes moderate antioxidant activity; (+) denotes weak antioxidant activity*

Scavenging activity on DPPH radical (Quantitative method)

The free radical scavenging activity of aqueous and ethanol extracts, were quantitatively assessed using the DPPH radical method adopted for spectrophotometry. Extracts that exhibited strong antioxidant activity were further pursued to quantify their antioxidant capacity using the DPPH method proposed by Brand-Williams, Cuvelier, and Berset (1995). Briefly, a 0.1mM solution of DPPH in ethanol was prepared and 1.0ml of this solution was added to 0.5ml of samples in different concentrations. After 20minutes, the absorbance was measured at 525nm. The DPPH radical-scavenging activity was calculated according to the following equation:

DPPH$^{\cdot}$ scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of the blank i.e no sample, DPPH solution only) and A_1 was the absorbance in the presence of the test compound.

Structural elucidation of the antioxidant principle from *Cassine transvaalensis*

^{13}C-NMR, ^{1}H-NMR, HSQC, and MS were used to elucidate the structure of the compound responsible for the antioxidant activity. The observed chemical shifts are as shown on table (1-1). From the spectroscopic data and also from the comparison with published data (Monache *et al*., 1992) compound **1** was identified as 4'-O-methyl-epigallocatechin.

Statistical analysis

All experiments were performed at least three times with each assay in triplicate with means for each assay recorded.

RESULTS AND DISCUSSION

DPPH radical scavenging activity of the extracts (Qualiatative)

The reduction of DPPH has been used to detect extracts with antioxidant activity, including those considered as free radical scavengers (Gamez *et al.*,1998).

Scavenging activity on DPPH radical (quantitative)

Fig. 1-6 shows the DPPH radical-scavenging activity of various solvent extracts of *Cassine transvaalensis*. As positive controls, epicatechin and L-ascorbic acid were also examined for DPPH radical scavenging activity. Chrysin, rutin and quercetin were run to explore the effect of presence or absence of –OH group on C or B ring of the flavonoid nucleus free radical scavenging capacity.

The scavenging capacity of flavonoids is determined by the substitution pattern (Cos *et al.*, 1998) on the flavonoid nucleus (Fig.5a).

4'-O-methyl-epigallocatechin was isolated from the crude water extract of *Cassine transvaalensis* (Fig. 1) using bio-assay guided fractionation. The structure of the isolated compound was confirmed by proton NMR and ^{13}C-NMR spectroscopy, and mass spectroscopy (Table 1 and Fig. 4). The structure was further confirmed by comparing the data with published work by Monache *et al.*, 1992 (Table 1).

Table 1:
DPPH radical scavenging activity by plant crude extracts

Name of plant (Family)	Bleaching of DPPH (Free radical scavenging activity)		
	Water extract	Ethanol extract	Isolated compound
Cassine transvaalensis (Celastraceae)	++++	++++	++++

Key: (++++)- strong anti-oxidant activity
This grading of activity was based on visual observation of the intensity of bleaching of DPPH solution.

Table 1:
^{13}C-NMR and ^{1}H-NMR 500MHz chemical shifts of the isolated compound **1** (methanol-d_4) and published work on 4'-O-methyl-(-)epigallocatechin (acetone-d_6) (Monache *et al*:1992)

C	Isolated compound 1		4'-O-methyl-(-)epigallocatechin	
	C-*Chemical shifts*	**H**-*chemical shifts*	**C**-*chemical shifts*	**H**-*chemical shifts*
2	79.7	4.77 *s*	79.17	4.84 *br s*
3	67.4	4.18 *br t*	66.78	4.23 *br t*
4	29.2	2.86 *dd* (17, 5.0) 2.72 *dd* (16.5, 3.5)	28.79	2.86 *dd* (16.5, 4.5) 2.73 *dd* (16.5, 3.2)
5	158.0	-	157.50[a]	-
6	96.4	5.93 *d* (3)	96.18	6.03 *d* (2.1)
7	157.7	-	157.45[a]	-
8	95.9	5.91 (3)	95.57	5.93 *d* (2.1)
9	157.2	-	156.81[a]	-
10	100.1	-	99.70	-
1'	136.6	-	136.16[b]	-
2', 6'	107.2	6.52 *s*	106.98	6.60 *s*
3', 5'	151.4	-	150.80	-
4'	136.1	-	135.40[b]	-
OMe	60.8	3.78 *s*	60.5	3.79 *s*

[a, b] Values in the same column may be interchanged

Figure 2:
TLC profile of 4'-O-methyl-epi-gallocatechin as a DPPH free radical scavenger; solvent system: (petroleum spirit: ethylacetate: water: methanol) 30:70:5:10. The TLC plate was sprayed with 0.2%DPPH solution in methanol

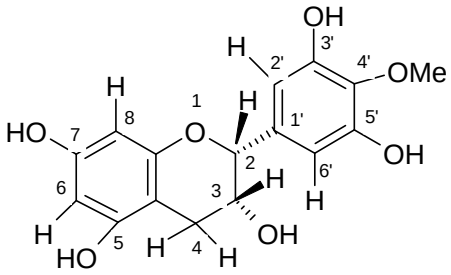
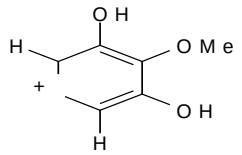


Figure 3:
Structure of 4'-O-methyl-(-) epigallocatechin (Compound **1**) Compound **1** crystalizes well as white needle-like crystals from chloroform.

Mass spec data*: EI+2.58e6*
m/z (100%) is found at 139.2 ($C_7H_7O_3$ requires 139.03948) is consistent with the loss of ring B.



$[M]^{+\bullet}$ *m/z* = 320.2 is consistent with $C_{16}H_{16}O_7$: ($C_{16}H_{16}O_7$ requires 320.08956)
$[M\text{-}H_2O]^+$ at 302.2 ($C_{16}H_{14}O_6$ requires 302.0790) consistent with loss of H_20

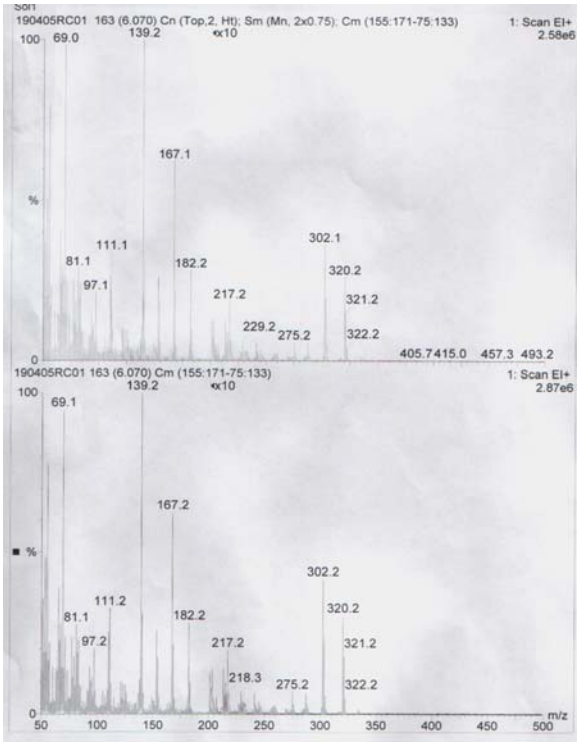


Figure 4:
Mass spectral data of 4'-O-methyl-(-) epigallocatechin

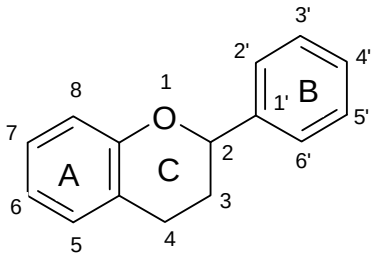


Figure 5a: The flavonoid nucleus

Figure 6, shows the DPPH radical-scavenging activity of ethanolic, water extracts of *Cassine transvaalensis* and 4'-O-methyl-epigallocatechin isolated from *Cassine transvaalensis* water extract. As positive controls, (-)epicatechin and L-ascorbic acid were also examined for DPPH radical scavenging activity. In order to investigate the effect of –OH groups of different flavonoids on DPPH radical scavenging, flavonoids that differed in hydroxyl substitution on the B or C-flavane ring were tested for their DPPH free radical scavenging capacity. Substitution pattern

for each tested flavonoid is as depicted in brackets and the structures are as shown in Figure 5b. 4'-O-methyl-epigallocatechin ((3,5,7,3',5'-OH,4'-OMe), quercetin, a flavonol (3,5,7,3',4'-OH), (-)epicatechin, a flavanol (3,5,7,3',4'-OH), rutin, a flavone (5,7,3',4'-OH, 3-rutinose), chrysin, a flavone (5,7-OH (lacking the 3, 3', 4'-OH)) were examined for their DPPH free radical scavenging capacity at concentrations (25-200µg/ml).

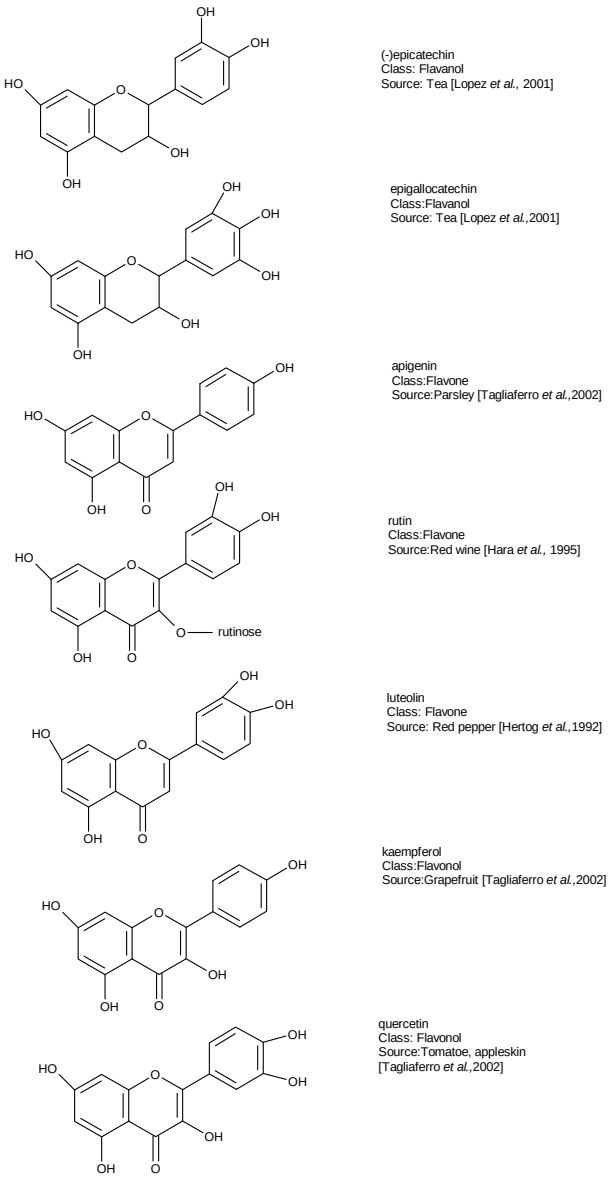


Fig. 5. Some antioxidants from plants

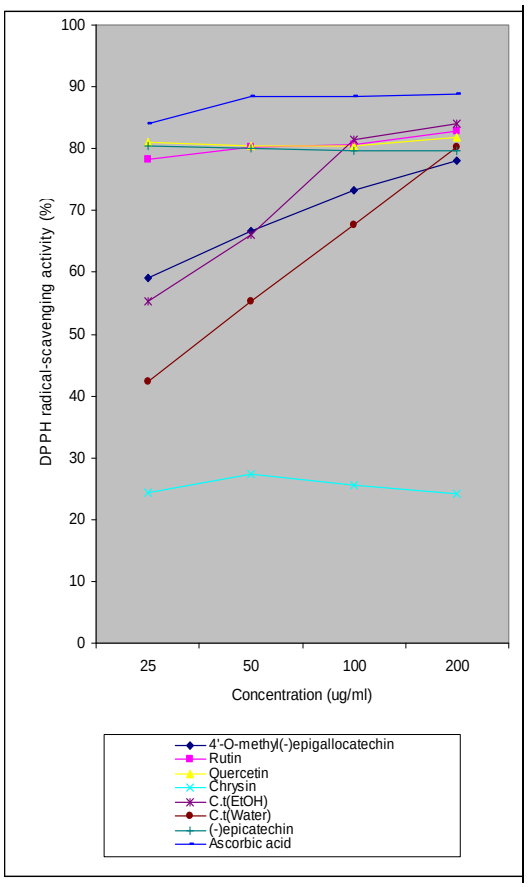


Figure 6:
Changes in DPPH radical scavenging activities according to different concentrations of C.t(Water) *C.transvaalensis* water extract; C.t(EtOH) *C.transvaalensis* ethanol extract; (-)epicatechin; L-ascorbic acid; chrysin, quercetin, rutin and the isolated compound 4'-O-methyl-epigallocatechin.

L-ascorbic acid showed the best results (~88%) through all the tested concentrations. Epicatechin, quercetin and rutin exhibited good scavenging activity (~80%). However, at 25µg/ml, rutin had a relatively lower scavenging activity (~78%) relative to either quercetin or (-)epicatechin. At all tested concentrations, chrysin, had the lowest DPPH radical scavenging activity (~26%).

The isolated compound had good scavenging activity (~70%) at 100µg/ml. As can be seen from Figure 1-6, this activity was lower than that of ascorbic acid, quercetin, rutin and epicatechin. At concentrations between 25-100µg/ml, significant differences were observed between compound

1(70%) and water extract (65%). However, as the concentration of the compound in the assay system increased, the differences in scavenging activities between compound and water extract became less significant.

Between 25-50µg/ml, the isolated compound had a greater scavenging activity than the ethanolic extract of *Cassine transvaalensis*, but, as the concentration of the ethanolic extract in the assay system increased, the extract exhibited a greater scavenging activity than the isolated compound. At all concentrations the ethanolic extract exhibited a greater scavenging activity than the water extract. At 100µg/ml and above, the scavenging activity of the ethanolic extract was greater than that of quercetin, rutin and epicatechin, This may suggest the presence of other constituents with free radical scavenging activity in the ethanolic extract.

The scavenging activity of the water extract though lower than that of rutin, L-ascorbic acid, quercetin, epicatechin, was greator than that of chrysin at all tested concentrations.

Previous studies of structure-activity relationships have generated several consistent lines of evidence supporting the role of specific structural components as requisites for radical scavenging (Tagliaferro *et al.,* 2002). Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity (Tagliaferro *et al.,* 2002). Antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure. Flavonoids with a 3-OH, and 3',4'-OH substitution are reported to be more potent than those lacking this substitution pattern (Tagliaferro *et al.,* 2002). Work done by Cos *et al.,* (1998), on structure-activity relationship of flavonoids as superoxide scavengers has also shown that the presence of a hydroxyl group at C-3' in ring B and at C-3 is associated with a high superoxide scavenging activity.

Steric obstruction of 3',4'-catechol structure by 4'-O-methylation significantly compromises antioxidant activity. This explains why 4'-O-methyl-epigallocatechin has a lower scavenging activity than (-)epicatechin, rutin or quercetin. The effect of 4'-O-methylation can also explain why 4'-O-methylation of quercetin to tamarixetin (3,5,7,3'-OH, 4'-OMe) decreases percentage inhibition of ferrous sulphate induced lipid peroxidation from 98% to -2.6% (Dugas *et al*.,2000).

The relatively low scavenging activity of rutin at 25µg/ml, could be attributed to the O-rutinose substituted 3-position of the C-ring. Removal of a 3-OH severely compromises scavenging ability (Acker *et al*., 1996).

Free radical scavenging of flavonoids is highly dependent on the presence of a free 3-OH (Burda and Oleszek, 2001). Quercetin exhibits a better scavenging activity than luteolin, supporting the role of the 3-OH group in free radical scavenging (Hirano *et al.,* 2001)

The findings of this experiment have shown that the radical scavenging activity of extracts or antioxidant constituents, might be mostly affected by the presence and position of the hydroxyl group.

Whilst the antioxidant activity of the water extract of *Cassine transvaalensis* can be attributed to 4'-O-methyl-epigallocatechin, the results of the ethanolic extract have shown possible contribution of additional components whose combined effect might confer a stronger antioxidant activity. The findings of this work support the use of *Cassine transvaalensis* in traditional medicine.

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Abbreviations:
NMR: Nuclear Magnetic Resonance
HSQC: Heteronuclear Single Quantum Correlation
HMBC:Heteronuclear Multiple Bond Correlation

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