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Antioxidant Activity of Flavonoids from the Leaves of *Tapinanthus pentagonia* (Loranthaceae)

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ABSTRACT

Phytochemical investigation of the crude methanolic extract of the leaves of *Tapinanthus pentagonia* (Loranthaceae) resulted in the isolation of four known flavonoids namely, quercetin 4'-methylether (1), 4'-methoxy-3',5,7-trihydroxyflavone (2), quercetin-3-O-rhamnoside (3) and quercetin 3-O-rhamnoside 4'-methylether (4). The structures of the isolated compounds were elucidated based on their 1D and 2D-NMR data. This is the first phytochemical study of that plant. The DPPH(2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, ABTS scavenging activity and ferric reducing antioxidant power (FRAP) were used to assess the antioxidant activities of the crude extract and three of the isolated compounds using catechin, ascorbic acid and gallic acid as standards, respectively. Results of the findings revealed that compound 3 exhibited the highest antioxidant activity in the three tests based on its IC₅₀ values followed by compound 4. Its IC₅₀ value was 0.024 mg/ml comparable to that of catechin (0.015 mg/ml) for the radical scavenging activity. In summary, compounds 1, 3 and 4 exhibited good antioxidant properties and reducing power compared to the crude methanolic extract.

Keywords: Loranthaceae, *Tapinanthus pentagonia*, flavonoids, antioxidant activity.

INTRODUCTION

The Loranthaceae (mistletoes) are flowering, chlorophyll and hemi-parasitic epiphytes that are located on the aerial parts of their hosts and are responsible for economic damage, ecological, technological and morphogenetic varied crops or the woody species parasitized [1]. They constitute the largest parasitic plant family with about 950 distributed species in 77 genera [2, 3] and are mostly characterized by persistent leathery leaves and brightly coloured inflorescence [4].

Mistletoes are known all over the world to cause damage to host plants. They pose serious threat to plantation by parasitizing cultivated and tended plants. They affect their host physiology leading to reduced growth, survival and reproduction. Despite their destructive nature to their host, the plants are very important in curative medicine. They are widely distributed throughout the world in the tropical and temperate zones and play an important role in the health of the local populations [5]. Mistletoes are now known as “cure all” and have been found beneficial for more than twenty health problems [6]. It is usual to prepare the leaves of a single species of mistletoe alone in medicine but more often, the leaves of two or more species which are thought to be the same but are only differentiated by the nature of their hosts are harvested and processed for use together. This is because the efficacy of drug preparations produced are thought to be dependent on the host plant(s) and that mistletoes harvested on certain hosts are suited for the treatment or cure of a particular health problem [7]. In Europe, the extracts are widely used in complementary and alternative cancer therapy. In Africa most particularly in Nigeria, several herbal preparations of the leaves and twigs are popularly used for the treatment of a variety of diseases such as diabetes and hypertension [8].

The taxonomy of these parasites especially in West Africa has been poorly studied. In Cameroon, 26 species were documented belonging to 7 genera: *Tapinanthus*, *Agelanthus*, *Phragmanthera*, *Englerina*, *Globimetula*, *Helixanthera* and *Viscum* [9]. They are all parasites of the xylem tissue and depend on their host for water, nutrients and some carbon compounds [1].

Tapinanthus is derived from the Greek word *tapeinos* meaning “low” or “humble” and *anthos* meaning flower. Some species are used to treat human and animal diseases such as cyst, sterility, convulsions, diabetes, arthritis, cancer, sexually transmitted disease [10, 11] and female sterility [12]. In Cameroon,

T. pentagonia is claimed to be used in the treatment of female infertility. Phytochemical screening on this plant parasite collected in Nigeria showed the presence of flavonoids, tannins, ellagic acid, proanthocyanidols, saponins, sterols, polyphenols, reducing sugars and terpenes. Various fractions of the extract exhibited good DPPH radical scavenging activity [10].

The present study is aimed at investigating the phytochemical and evaluating the *in vitro* antioxidant activity of the methanolic extract of the leaves of *Tapinanthus pentagonia* using free radical scavenging DPPH, ABTS and the reduction power as part of our ongoing program of searching for bioactive natural products from Cameroonian medicinal plants.

The use of synthetic antioxidant molecules is currently being criticized because of the potential toxicological risks. Now, new plants, sources of natural antioxidants, are being sought [13]. Polyphenols are natural compounds widely used in the plant kingdom in particular because of their beneficial effects on health. Their role as natural antioxidants is attracting more and more interest in the prevention and treatment of cancer, inflammatory and cardiovascular diseases. Scientific research has been developed for the extraction, identification and quantification of these compounds from different sources such as agricultural and horticultural crops or medicinal plants [14].

Antioxidant is a substance that even at low concentration delays and prevents the oxidation of the substrate [15]. The most known antioxidants are β -carotene, ascorbic acid and tocopherol. Indeed, most of the synthetic or naturally occurring antioxidants have hydroxy phenolic groups in their structures and the antioxidant properties are attributed in part to the ability of these natural compounds to trap free radicals such as hydroxyl radicals (OH \cdot) and superoxide's (O $_2^{\cdot-}$) [16].

It is evident that Loranthaceae play an important role in the traditional pharmacopoeia of local populations. However, there are some limitations such as the lack of awareness of their chemical composition. Therefore, additional phytochemical and pharmacological studies are required to isolate their active ingredients [5].

MATERIALS AND METHODS

General experimental procedures

Column chromatography (CC) was performed with silica gel (200–425 mesh particle size) and thin layer chromatography (TLC) on pre-coated silica gel on aluminum sheets. The NMR data were recorded with a Bruker spectrometer [^1H NMR (500 MHz) and ^{13}C NMR (125 MHz)] with tetramethylsilane (TMS) as standard.

Sample collection

The leaves of *Tapinanthus pentagonia* (Loranthaceae) growing on avocado trees were collected in Yaounde, Centre region of Cameroon in December 2009. The plant was identified with the help of a botanist of the Institute of Medical Research and Medicinal Plant Studies (IMPM). A voucher specimen was deposited at the National Herbarium of Yaounde, Cameroon under the number No. 67002/HNC.

Chemicals and reagents

For the bioassay, DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS(2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), iron III chloride (FeCl $_3$), potassium persulfate (K $_2$ S $_2$ O $_8$), potassium ferricyanide K $_3$ Fe(CN) $_6$, trichloroacetic acid (TCA), gallic acid, ascorbic acid, quercetin, tannic acid were purchased from Sigma Aldrich, St Louis, USA and methanol from local suppliers.

Extraction and isolation

The leaves of *T. pentagonia* were air-dried and ground into fine powder in a grinder. The ground powder was then packed and stored into a

labeled zip locked polythene bag at room temperature before being transported to the Phytochemistry Laboratory for studies. 4.156 kg of the leaves were extracted at room temperature with 11 L of methanol. After 48 hours, the solution was filtered and concentrated with a rotavapor under *vacuum* at 40 $^\circ$ C and then dried in a ventilated oven at 50 $^\circ$ C for 48 hours to yield 250g of a solid residue.

Part of the crude extract (60 g) was subjected to open column chromatography with silica gel as adsorbent. Elution was performed with hexane/ethyl acetate and ethyl acetate/methanol in increasing polarity.

A total of 140 fractions (250 ml each) were collected, concentrated *in vacuo* in a rotavapor and their compound profiles monitored by using analytical TLC plates. Similar fractions were combined giving a total of 12 fractions (Mobile phase: CH $_2$ Cl $_2$ or CH $_2$ Cl $_2$ /EtOAc, 1:1).

Compound 1, (15mg) was isolated as a yellow powder from Fr6 - Fr8 of the main fractionation column eluted with 60% EtOAc in hexane.

The fractions of the main column eluted with 90%EtOAc in hexane were combined and the solvent evaporated using the rotavapor. The solid formed was re-crystallized using ethyl acetate resulting to a yellow compound 2 (8 mg).

Part of the methanol extract was treated with a mixture of CH $_2$ Cl $_2$ -MeOH-H $_2$ O (2:2:1). The dichloromethane portion was dried with MgSO $_4$ and concentrated *in vacuo* using a rotavapor. The extract obtained was subjected to a silica gel column eluted with hexane/EtOAc of increasing polarity. Fractions with interesting profile on TLC were then passed through the Chromatotron with 80% EtOAc in hexane. A total of 24 fractions (30 ml each) were collected. Compound 3, (18 mg) was obtained from Fr21- Fr23 as yellow crystals.

Fr5-Fr18 was passed through the Chromatotron with 80% EtOAc in hexane to give compound 4 as yellow crystals.

Antioxidant activity assessment

DPPH Radical Scavenging assay

The ability of an extract to scavenge the radical DPPH was evaluated using the method of Blois [17] with some modifications. 2 ml of a methanolic solution of DPPH (0.1 mM) freshly prepared followed by the addition of 0.5 ml of *T. pentagonia* extract and compounds (0.125, 0.0625, 0.03125, 0.015625 mg /ml) were mixed and incubated for 30 minutes in the dark at room temperature. The optical density was recorded on a spectrophotometer at 517 nm against the blank consisting of 0.5 ml of methanol and 2 ml of DPPH solution. Gallic acid was used as standard and prepared at concentrations of 0.125, 0.0625, 0.03125 and 0.015625 mg/ml. The test was done in triplicate and the percent inhibition of the DPPH radical was calculated according to the following formula:

$$\% \text{inhibition DPPH} = (\text{Absblank} - \text{Abs sample} / \text{Abs blank}) * 100$$

The IC $_{50}$ of the various extracts and compounds were calculated by plotting a graph of percentage inhibition against logarithm of concentration and then extrapolating from the 50% inhibition to obtain the IC $_{50}$ values using SPSS software.

ABTS radical scavenging assay [18]

This is one of the most widely used methods for determining the antioxidant activity of plant extracts. It consists in following the kinetics of discoloration of the ABTS $^+$ ion. ABTS (2,2'-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)) was prepared by mixing 0.0384 g of ABTS and 0.00662 g of potassium persulfate (K $_2$ S $_2$ O $_8$) with 10 ml of distilled water. The mixture was kept in the dark at room temperature for 16h before use. For the analysis, the ABTS solution was diluted with methanol and the absorbance adjusted to 1.400 (\pm 0.02) at 734 nm

and stable at 30°C (initial OD). To determine the scavenging activity, 1 ml of this diluted ABTS solution and 0.25ml of the extract or isolated compounds (0.125, 0.0625, 0.03125 and 0.015625 mg/ml) were mixed and incubated for 15 min at room temperature. Absorbance reading was taken at 734 nm. Ascorbic acid was used as an antioxidant reference at the same concentrations as the extracts. The percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition ABTS} = [(\text{Abs blank} - \text{Abs sample}) / \text{Abs blank}] \times 100$$

The IC₅₀ of the various extracts were calculated by plotting a graph of percentage inhibition against logarithm of concentration and then extrapolating from the 50% inhibition to obtain the IC₅₀ values using SPSS software.

Ferric Reducing Antioxidant Power (FRAP) assay

0.2 ml of the extract at different concentrations (from 0.007 to 2.5 mg/ml) was mixed with 0.5 ml of a 0.2 M phosphate buffer solution (pH 6.6) and 0.5 ml of a solution of potassium ferricyanide (1% K₃Fe(CN)₆). The whole was incubated in a water bath at 50°C for 20 minutes. Thereafter, 0.5 ml of 10% trichloroacetic acid was added to stop the reaction and the tubes were centrifuged at 3000 rpm for 10 min. An aliquot (0.5 ml) of supernatant was combined with 0.5 ml of distilled water and 0.1 ml of 0.1% aqueous FeCl₃ solution. The absorbance of the reaction was read at 700 nm against a similarly prepared blank. The standard was represented by a solution of ascorbic acid whose absorbance was measured under the same conditions as the samples. The ferric reducing antioxidant power was expressed in mg AAE per gram of extract. In the present study, the results of the Ferric Reducing Antioxidant Power (FRAP) of the methanolic extract and compounds from *T. pentagonia* are presented in Table 3 and are expressed in mg.GAE/g of extract using the calibration curve of gallic acid ($y = 6.124x + 0.2757$, $R^2=0.9995$).

RESULTS

Phytochemical studies

The methanolic extract of the leaves of *Tapinanthus pentagonia* was fractionated by open silica gel column and Sephadex LH-20 chromatography, medium pressure liquid circular chromatography using Chromatotron to yield compounds 1–4 (Figure 1). They were identified as quercetin 4'-methylether (1) [19], 4'-methoxy-3',5,7-trihydroxyflavone (2) [20], quercetin-3-O-rhamnoside (3) [21] and quercetin 3-O-rhamnoside 4'-methylether (4) [22].

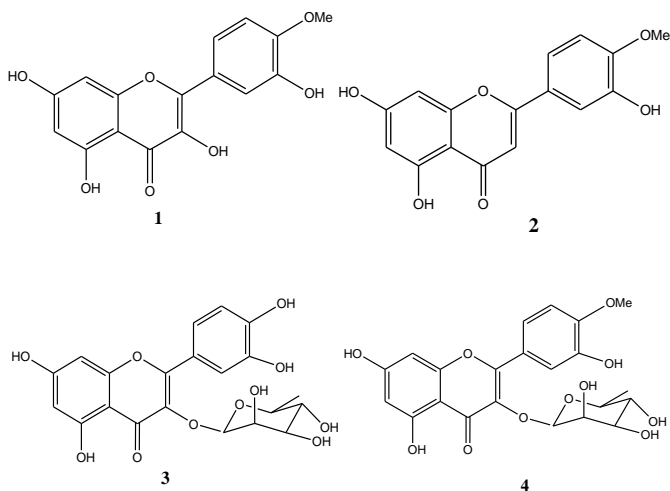


Figure 1: Structures of isolated compounds

Their structures were determined by analysis of their NMR data and comparison with those reported in literature. It is worth mentioning that this is the first report on the four compounds from *T. pentagonia* to the best of our knowledge of flavonoids from the *Tapinanthus* genus.

Antioxidant Activity

A wide variety of antioxidant assays are used when determining the antioxidant activity of plant extracts and isolated compounds. In this study, three different methods were used to assess the antioxidant activity of *T. pentagonia* (leaves) and pure compounds, namely, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, ABTS scavenging activity and FRAP (ferric reducing antioxidant power).

Radical DPPH Scavenging activity [23]

The reduction of DPPH radical by antioxidants is evaluated by the decrease in absorbance at 517 nm. In this study, the ability of the extract and isolated compounds to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated. The results presented in Table 1 show that compounds isolated from *T. pentagonia* have a high percentage of inhibition that increases in a dose-dependent manner. The largest percentage inhibition was found for compound 3 with a percentage inhibition of DPPH of 90.15% at 0.125 mg/ml. Compounds 1, 4 and the methanolic extract (TP) respectively have a percentage of inhibition of 60.62, 68.55 and 54.56% at the same concentration of 0.125 mg/ml significantly lower compared to catechin which has 88.43% and 3 ($p < 0.001$). The IC₅₀ values, expressing the effective concentration of the antioxidant extract required for entrapment and the 50% reduction of dissolved DPPH in methanol, were calculated. The results show an IC₅₀ for the crude extract and those of compounds 1 and 4 are higher than that recorded for catechin (0.015 mg/ml, Table 1, Figure 2).

Table 1: Percent inhibition of the DPPH radical and IC₅₀ values of crude extract and compounds 1, 3 and 4.

Concentration (mg/ml)	TP	1	3	4	Catechin
0.125	54.65 ^a	60.62 ^a	90.15	68.55 ^a	87.43
0.0625	44.85	39.87	85.55	37.14	81.43
0.03125	41.81	30.25	53.55	35.89	67.97
0.015625	38.73	25.94	37.96	33.94	50.10
IC ₅₀ (mg/ml)	0.087	0.086	0.024	0.066	0.015

^a $p < 0.001$ significantly lower compared to ascorbic acid. TP: methanolic extract of *T. pentagonia*.

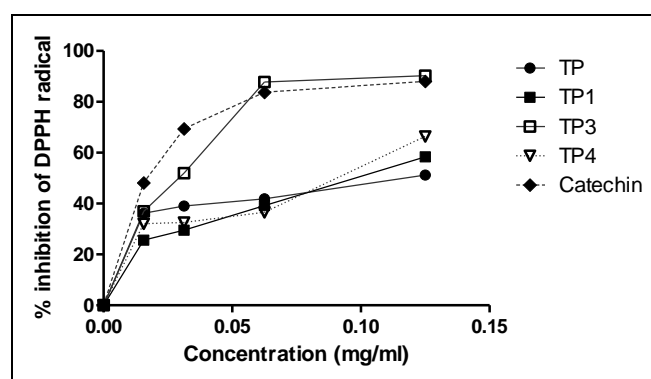


Figure 2: Inhibition percentage of DPPH radical of *T. pentagonia* and isolated compounds

Inhibition of ABTS radical

It is one of the most used tests for determining the antioxidant activity of plant extracts. Methanolic extract (TP) of *T. pentagonia* has a significantly lower ($p < 0.05$) effect on ABTS inhibition activity (17.73%) compared to that of ascorbic acid (96.37% at the concentration of 0.0125 mg/ml). The IC₅₀ value of compound 3 was significantly comparable to that of ascorbic acid used as standard (Table 2, Figure 3).

Table 2: Inhibition percentage of ABTS radical and IC₅₀ values of compounds 1, 3, 4 and the methanolic extract of *T. pentagonia*

Concentrations (mg/ml)	TP	1 3	4	Ascorbic acid
0.125	17.73 ^a	55.58 ^a	51.06 ^a	96.37
0.0625	15.44 ^a	20.83 ^a	35.70 ^a	53.73
0.03125	12.84	26.33	14.12	27.14
0.015625	10.27	18.68	18.51	15.23
IC ₅₀ (mg/ml)	0.951	0.080	0.122	0.046

^ap<0.001 significantly lower compared to ascorbic acid. TP: methanolic extract of *T. pentagonia*.

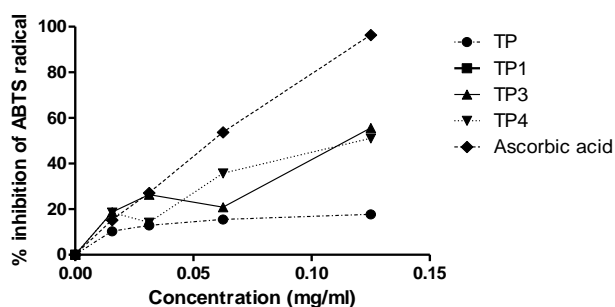


Figure 3: Inhibition percentage of ABTS radical of *T. pentagonia* and compounds.

Ferric Reducing Antioxidant Power (FRAP)

In this assay, the compounds with reduction potential react with potassium ferricyanide (Fe³⁺) to form potassium ferrocyanides (Fe²⁺), which then react with ferric chloride to form ferric ferrous complex that is greenish in colour [24]. In the present study, the results of the Ferric Reducing Antioxidant Power (FRAP) of the methanolic extract and compounds from *T. pentagonia* are presented in Table 3 and are expressed in mgGAE/g of extract using the calibration curve of gallic acid ($y = 6.124x + 0.2757$, $R^2=0.9995$). The strongest ferric reducing ability was shown by compound 3 (4.46mg GAE/g) followed by compound 4 (4.19 mgGAE/g). The lowest activity was obtained with compound 1 (0.57 mg GAE/g).The methanolic extract (TP) was inactive in the FRAP assay. The study was assessed at the concentration of 0.5 mg/ml.

Table 3: Ferric reducing antioxidant power (FRAP) of compounds 1, 3, 4 and methanolic extract (TP) of *T. pentagonia*

	TP	1	3	4	Gallic acid
FRAP (mg AAE/g extract)	0.00	0.57±0.04	4.46±0.03	4.19±0.00	8.51±0.05

Values represent mean± SD of triplicate readings, n = 3; AAE: Ascorbic acid equivalent

DISCUSSION

Medicinal plants used in traditional medicine are particularly interesting for investigation of their antioxidant properties. According to Halilu *et al.* [24], biologically active compounds isolated from plants have played enormous roles in the development of new drugs. These compounds which are secondary metabolites have diverse chemical structures. Species of Loranthaceae constitute rich sources of phytochemical constituents and are known to have potential medicinal properties [25, 26]. Previous studies [13, 24, 27, 28] have reported the antioxidant capacity of some *Tapinanthus* species. However, there were no studies regarding antioxidant potential of chemical constituents of *T. pentagonia*.

DPPH is a stable radical commonly used to determine the antioxidant activity of various compounds. The method of DPPH radical scavenging is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant, inducing a color change from purple to

yellow at 517 nm [29]. Results of our findings reveal that compound 3 has a higher antioxidant activity followed by compound 1 and 4 and then the methanolic extract (TP) with an IC₅₀ of 0.024 mg/ml comparable to the IC₅₀ of catechin (0.015 mg/ml). Compounds 1, 3 and 4 are phenolic compounds. This class of compounds was previously identified through the phytochemical screening carried out by Samba *et al.* [10] on the leaves of *Tapinanthus pentagonia*. It is known that phenolic compounds are the main contributors to antioxidant properties of plants. They exert their antioxidant properties by scavenging free radicals, chelating metals, reducing action and inhibition of lipid peroxidation [30]. Polyphenolic compounds are capable of donating electrons or transferring hydrogen atom to neutralize free radicals to scavenge the free radicals and to help maintain healthy body [31]. Similar results were obtained by Samba *et al.* [10] wherein the methanolic extract of fruits and leaves of *T. pentagonia* showed a powerful antioxidant effect.

Total antioxidant using ABTS is another test for the evaluation of the antioxidant properties of compounds from plants. The results presented in the present study reveal that the inhibitory percentage of ABTS radical is higher in compound 3 followed by compound 4. However, the antioxidant activity of these compounds is lower compared to ascorbic acid (0.046 mg/ml).The antioxidant potentials of plants extracts can be expressed on the basis of their IC₅₀ values. The lower the IC₅₀ value the more potent the extract is as antioxidant or a free radical scavenger. Antioxidant activities of phenolics compounds may also exhibit their radical scavenging ability and redox properties by acting as reducing agents, hydrogen donors and singlet oxygen quenchers [28].

The reducing power of a compound may count as a strong indicator of its antioxidant potential. The reducing power has been applied in most of the methods available for the determination of antioxidant potential [32]. According to the results obtained, the reducing power of the compounds is higher for compound 3 followed by compounds 1 and 4. The methanolic extract (TP) did not show reducing power. Ferric reducing antioxidant power of phytochemical compounds is actively dependent on the antioxidant activity. The compounds from *T. pentagonia* being phenolic compounds show a strong antioxidant power. In the FRAP assay, Fe³⁺ions accept an electron to become Fe²⁺ which is responsible for the resulting green-blue colour. The higher intensity of the colour, the more the reducing power. There is a direct correlation between antioxidant activity and reducing power of the components of some plants [16, 29]. The presence of antioxidants in the sample will result in the reduction of Fe³⁺ to Fe²⁺ by donating an electron which is an important mechanism of phenolic antioxidant action [29].

Compound 2 (4'-methoxy-3',5,7-trihydroxyflavone) was not tested due to limited amount.

Flavonoids are able to chelate free radicals immediately by donating a hydrogen atom or by single-electron transfer. They also inhibit free radical generating enzymes such as xanthine, lipoxygenase [24]. Several studies [33, 34, 35] have shown that the presence 5-hydroxyl groups in the B- ring especially in the *ortho* position is of utmost significance to the antioxidant activity of flavonoids as well as the total number of hydroxyl groups on the entire molecule through the formation an intramolecular hydrogen bonding and the ability to give out proton easily. In addition, studies by Zheng *et al.* [36] also demonstrated through computational studies that the presence of sugar on the B-ring and C-ring of flavonoids contributes positively to their antioxidant activity. Furthermore, the presence of a methyl group in the structure of flavonoids has been shown to decrease their antioxidant activity [37]. These flavonoids isolated from *T. pentagonia* also possess the distinctive chemical structures for the antioxidant activities of flavonoids such as the presence of the hydroxyl groups in the B-ring and the C2-C3 unsaturated bond combined with the C-4 carbonyl group in the C-skeleton. This explains why all three compounds tested showed interesting antioxidant activities. The highest antioxidant activity of 3could be explain by the fact that it best fits into the

structural features responsible for the antioxidant properties of flavonoids compared to 4 and 1 and it lacks a methyl group in its structure. Compounds 1 and 4 are similar in structure but for the fact that 4 possess a sugar at position 3 unlike compound 1 giving it a better antioxidant activity than the latter.

CONCLUSION

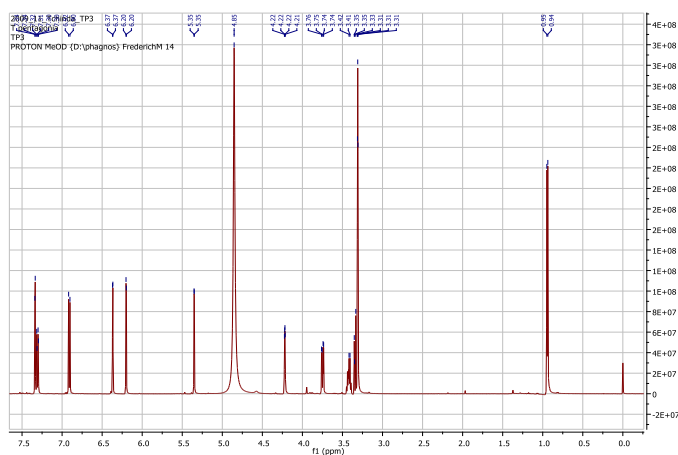
The present study shows that the methanolic extract of the leaves of Cameroonian *Tapinanthus pentagonia* contains polyphenols. Three of the four isolated compounds 1, 3 and 4 exhibited good antioxidant properties and reducing power compared to the methanolic extract. This could be explained by the fact that these compounds are phenolic compounds. Not much information is readily available on the chemistry of many African mistletoes. This work represents an extensive phytochemical study on Mistletoe which will contribute to the chemotaxonomy of this important group of plants. The results obtained in the course of this work show that *T. pentagonia* is rich in antioxidant compounds. Antioxidants play an important role in various diseases. There could be a link between the antioxidant activity of *T. pentagonia* and its use in traditional medicine for the treatment of several human ailments.

Acknowledgements

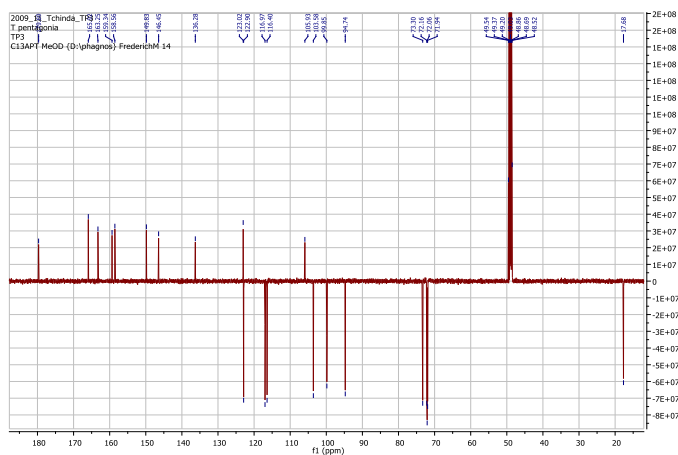
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SUPPLEMENTARY MATERIALS

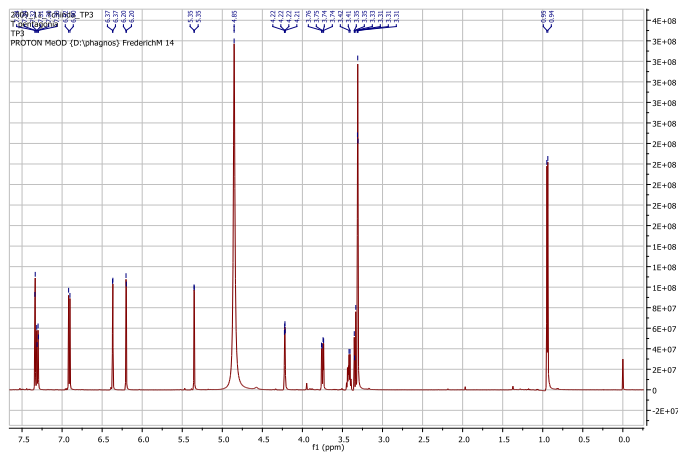
¹H and ¹³C NMR spectra of compounds 1-4 are included within the supplementary information file.



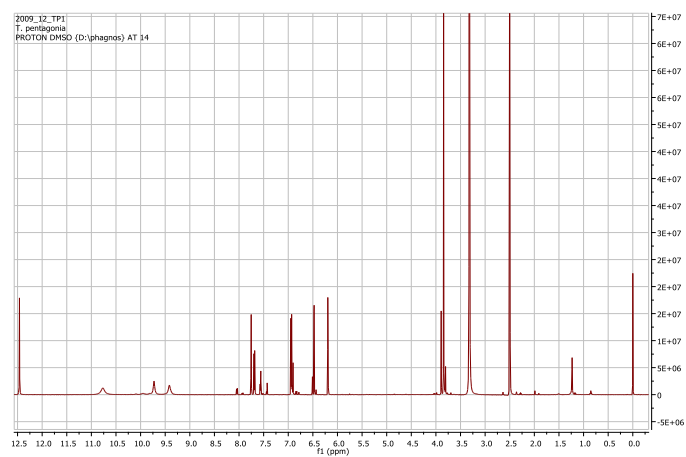
¹H NMR spectrum of compound 2



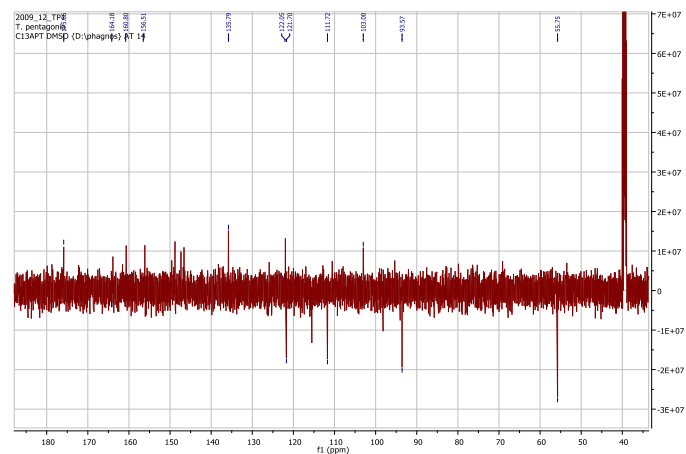
¹³C NMR spectrum of compound 2



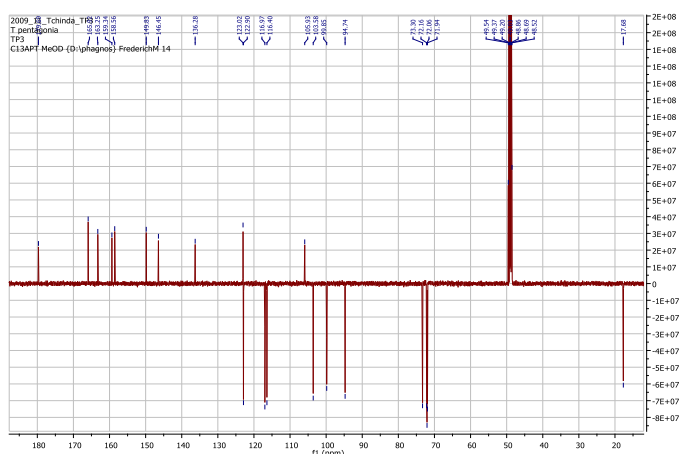
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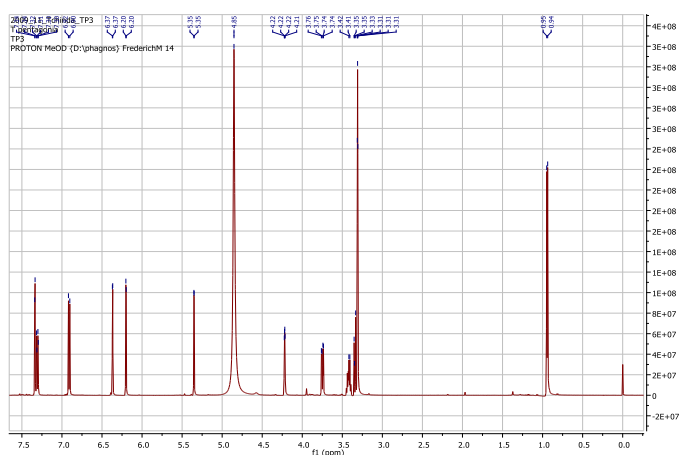
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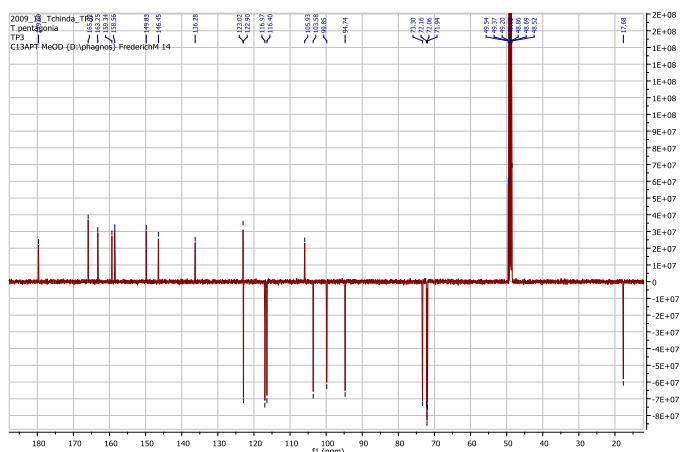
¹³C NMR spectrum of compound 1



¹³C NMR spectrum of compound 3



¹H NMR spectrum of compound 4



¹³C NMR spectrum of compound 4

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