

Identification, Quantitative Determination, and Antioxidant Properties of Polyphenols of Some Malian Medicinal Plant Parts Used in Folk Medicine

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1. Introduction

In biological systems, during the cellular respiration, reactive oxygen species (ROS) like hydroxyl radical ($\bullet\text{OH}$), superoxide anion ($\bullet\text{O}_2^-$) and hydrogen peroxide (H_2O_2) are generated, as the natural consequence of oxidation reactions (Tarnawski et al., 2005). Reactive oxygen species (ROS) damage living cells causing lipid, protein, and DNA oxidation (Shukla et al., 2010). They are involved in the development of various diseases such as diabetes, rheumatic disorders (Luximon- Ramma et al., 2002), aging, cancer, cardiovascular or neurodegenerative disorders (Ju et al., 2004; Tarnawski et al., 2005), malaria and gastric ulcer (Gülçin et al., 2006).

The interest in searching natural antioxidants has recently increased. These natural products could be used in food or in medicinal materials to replace synthetic antioxidants which are about to be restricted owing to their side effects such as carcinogenesis (Gülçin et al., 2006). Many medicinal plants contain large amounts of antioxidants, such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, in quenching singlet and triplet oxygen, or in decomposing peroxides. The compounds that are responsible of antioxidant activity could be used for the prevention and treatment of free radical-related disorders (Gomez-Caravaca et al., 2006). Indeed, the consumption of antioxidants prevents different diseases such as neurological degeneration, inflammatory disorders, coronary diseases, aging and cancers (Djeridane et al., 2006). Hence, the studies on natural antioxidants have gained increasingly greater importance.

A large number of different plants have been studied as new sources of natural antioxidants (Cakir et al., 2003; Lee et al., 2000; Kumaran & Karunakaran, 2007; Muanda et al., 2009). For the first time, we report here the antioxidant properties of extracts from the following six Malian folk medicine plants, which were previously studied for their biological activities:

Anogeissus leiocarpus (DC.) Guill. et Perrot (Combretaceae), *Cissus populnea* Guill. et Perr. (Vitidaceae), *Mitragyna inermis* (Willd.) O. Ktze. (Rubiaceae), *Terminalia macroptera* Guill. et Perrott (Combretaceae), *Vepris heterophylla* R. Let. (Rutaceae) and *Zizyphus mucronata* Willd. (Rhamnaceae). These plants were selected for their traditional used in the treatment of inflammatory diseases such as: malaria, oedema, arthritis, rheumatism, ulcer, gingivitis, conjunctivitis (Burkill, 2000; Malgras, 1992; Arbonnier, 2002; Inngjerdingen et al., 2004). Vonthron-Sénécheau et al., (2003) reported the *in vitro* antiplasmodial activity of the extracts of the leaves of *Anogeissus leiocarpus*. Geidam et al., (2004) reported evidence-proved similar effects of the aqueous stem bark extract from *Cissus populnea* on some serum enzymes in alloxane induced diabetic rats. They have attributed hypoglycaemic properties to these extracts. Aqueous extract from *Mitragyna inermis* has been used by traditional healers for the treatment of various diseases, particularly for hepatic illness, malaria and hypertension. Recently, studies by Ouédraogo et al., (2004) demonstrated the hypotensive, cardiotropic and vasodilatory properties of bark aqueous extract from *Mitragyna inermis*. To identify new antimalarial compounds, Conrad et al. (1998) selected *Terminalia macroptera* for an antiplasmodial screening. Moulis et al., (1994) studied the volatile constituents of the leaves of *Vepris heterophylla*. They found that among thirty-three compounds – that were identified by capillary GC – the main constituents were geijerene and pregeijerene. Recently, Mølgaard et al., (2001) reported good activity of the extracts of root bark from *Zizyphus mucronata* which were tested *in vitro* against tapeworms and schistosomules.

To our knowledge, there are no previous reports concerning *in vitro* antioxidant activities of these plant part extracts. The purposes of this study were to determine the total phenolic and the total flavonoid contents, to evaluate their antioxidant activities using 2, 2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) tests, and finally to identify and to quantify some polyphenolic compounds by using a RP-HPLC coupled to an UV detector.

2. Materials and methods

2.1 Plant material

Professor N'Golo Diarra performed the plant taxonomy in the "Departement of Traditional Medecine" in Bamako, and voucher specimens were deposited in its herbarium as *Anogeissus leiocarpus* (DC.) Guill. et Perrot (Combretaceae) Ref. N° 1559, *Cissus populnea* Guill. et Perr. (Vitidaceae) Ref. N°1368, *Mitragyna inermis* (Willd.) O. Ktze. (Rubiaceae) Ref. N°1394, *Terminalia macroptera* Guill. et Perrott (Combretaceae) Ref. N°1617, *Vepris heterophylla* R. Let. (Rutaceae) Ref. N°2444 and *Zizyphus mucronata* Willd. (Rhamnaceae) Ref. N°2499. The plant material was collected around the district of Bamako in December 2005.

2.2 Apparatus

The HPLC analyses were performed with a Waters 600E coupled to a Waters 486 UV visible tunable detector (SPD-M10Avp) and a Reverse Phases C18 symmetry analytical Alltech Intertsil ODS- 5 μm 4.6mm \times 150 mm column. In addition, spectrophotometer analyses were carried out with a Cary 50 scan UV- Visible apparatus (UV Mini 1240).

2.3 Chemical reagents

Standards: catechin and gallic acid, 3,4 dihydroxybenzoic acid (protocatechuic acid), chlorogenic acid, rutin were purchased from Across Organics (France). *p*-coumaric acid, isovitexin and quercetin 3- β -D-glucoside were obtained from Fluka Chemical Company (France).

Aluminium chloride (AlCl₃), ascorbic acid, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), PBS buffer, 2,2'-azobis (2-methylpropionamide) dichloride (AAPH), Folin-Ciocalteu's phenol reagent, sodium carbonate (Na₂CO₃), caffeic acid and sodium nitrite (NaNO₂), stable free radical DPPH were purchased from Sigma Chemical Company (France). All commercial standards and reagents were of the highest analytical grade.

2.4 Preparation of extracts

Each plant material was dried in a dark ventilated room for 5–7 days. The different parts of the plants (leaves, root barks, and stem barks) were ground to powder and sifted in a sieve (0.750 μ m).

The extraction of the samples was performed by the ultrasound-assisted method (Kim et al., 2002, 2003). The air-dried plant material (10 g) was extracted using 100 mL of 80% aqueous methanol, the mixture of freeze-dried powder and 80% aqueous methanol was sonicated for 20 min with continual nitrogen gas purging. The mixture was filtered through Whatman N^o2 filter paper and rinsing with 50 mL of 100% methanol. The extraction of the residue was repeated using the same conditions and the two filtrates were combined and transferred into a 1 L evaporating flask with an additional 50 mL of 80% aqueous methanol. The solvent was evaporated using a rotary evaporator at 40 °C. The remaining extract concentrate was first dissolved in 50 mL of 100% methanol and diluted to a final volume of 100 mL using distilled deionized water (ddH₂O). The mixture was centrifuged at 1500g for 20 min and stored at -4°C until analyses were performed.

2.5 Determination of the total phenolic and of the total flavonoid contents

The concentration of total phenolics was measured by the method described by (Kim et al., 2003). Briefly, an aliquot (1 mL) of appropriately diluted extracts or standard solutions of gallic acid was added to a 25 mL volumetric flask containing 9 mL of ddH₂O. A reagent blank was prepared using ddH₂O. One milliliter of Folin & Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7% Na₂CO₃ solution were added and the solution was then immediately diluted to volume (25 mL) with ddH₂O and mixed thoroughly. After an incubation of 90 min at 23 °C, the absorbance versus prepared blank was read at 750 nm (Cary 50 Scan UV-Visible apparatus). Total phenolic contents of plant parts are expressed as mg of gallic acid equivalents (GAE) / g dry weight. All samples were analyzed at least in triplicate.

Total flavonoids were measured by a colorimetric assay that was developed by Zhishen et al. (1999). We can add to a 10 mL volumetric flask containing 4 mL ddH₂O either 1 mL of aliquot of appropriately diluted sample or 1 mL of a standard solution of catechin. At zero time, 0.3 mL 5% NaNO₂ was added to the flask. After 5 min, 0.3 mL 10% AlCl₃ was added.

After 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the reaction flask had to be diluted to volume by the addition of 2.4 mL of ddH₂O and thoroughly mixed. Absorbance of the mixture was determined at 510 nm (Cary 50 Scan UV-Visible apparatus) versus prepared water blank. Total flavonoids of plant parts are expressed as mg / g dry weight of catechin equivalents (CE). Samples were analyzed at least in triplicate.

2.6 Determination of total antioxidant activity

Various methods have been introduced for the measurement of the total antioxidant capacity (Delgado-Andrade et al., 2005; Gülçin et al., 2006). In this study antioxidant activity was estimated by the method previously described (Kim et al., 2002, 2003). The *in vitro* antioxidant activities have been determined in two antioxidant tests. Among the different methods permitting to evaluate the antioxidant activities, these two simple stable radical chromogens have a high level of sensitivity and allow the analysis of a large number of samples in a timely fashion (Kim et al., 2002). It was reported that a single method is not enough to evaluate the antioxidant capacity of most of the complex natural products (Ozgen et al., 2006). Antioxidant capacity is expressed as mg of vitamin C equivalent (mg VCE) per g dry weight.

2.7 ABTS radical anion scavenging activity

ABTS radical anions were used according to the method of (Kim et al., 2003). In brief, 1.0 mM of 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH), a radical initiator, was mixed with 2.5 mM ABTS in phosphate-buffered saline (pH 7.4) and the mixed solution was heated in a water bath at 68 °C for 13 min. The resulting blue-green ABTS solution was adjusted to the absorbance of 0.650 ± 0.020 at 734 nm with additional phosphate-buffered saline. 20 µl of sample were added to 980 µL of the ABTS radical solution. The mixture incubated in a 37°C water bath under restricted light for 10 min. A control (20 µL 50% methanol and 980 mL of ABTS radical solution) was run with each series of samples. The decrease of the absorbance at 734 nm was measured (Cary 50 Scan UV-Visible apparatus) at an endpoint after 10 min. Total antioxidant capacity of plant parts is expressed as mg / g of dry weight of vitamin C equivalents (VCEAC). The radical stock solution had to be freshly prepared and all measurements of the tested samples were repeated at least three times.

2.8 DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to the method of Kim et al., (2002). The DPPH radical (100 µM) was dissolved in 80% of aqueous methanol. The plant extract solutions, 0.1 mL, were added to 2.9 mL of the methanolic DPPH solution and the mixture was vigorously shaken and was kept at 23 °C in the dark for 30 min. The decrease of the absorbance of the resulting solution was monitored at 517 nm (Cary 50 Scan UV-Visible apparatus) after 30 min. A control, which consists of 0.1 mL of 50% aqueous methanol and 2.9 mL of DPPH solution, was prepared. The DPPH radical scavenging activity of plant extracts is expressed as mg/g of dry weight of vitamin C equivalents (VCEAC). This measure was taken after 30 min reaction time. The radical stock solution had to be daily prepared and the tests were repeated at least three times.

2.9 HPLC analysis

The HPLC analyses were conducted with a Water 600 Pump apparatus. This apparatus was equipped with a quaternary solvent delivery system, a Rheodyne injector with 20 μ L sample loop and a UV detector Waters 486 Tunable which was fixed at 280 nm. Throughout this study, Alltech Intertsil ODS-5 C18 reversed phase column (150 mm, 4.6 mm, 5 μ m particle size) was used. The flow rate of the mobile phase was of 1 mL / min and the gradient elution was adapted from (Nakatani et al., 2000; Bouayed et al., 2007). The solvent composition and the gradient elution program are reported in the table 1.

Times (min)	%A	%B	%C
0	100	0	0
5	65	12	23
11	0	15	85
29	0	22	78
36	0	25	75
42	0	25	75
55	0	35	65
60	0	50	50
70	0	100	0
75	100	0	0

Solvent composition: A =50 mM NH₄H₂PO₄ at pH 2.60; B = 80% acetonitrile, 20 %A and C = 200 mM O-phosphoric acid at pH 1.50

Table 1. HPLC solvent gradient elution program

Standards of five phenolic acids and two flavonoids were dissolved in 50% MeOH to make a concentration of 0.5; 0.25; 0.125 and 0.10 mg/mL. The plant part extracts and standards solutions were filtered through 0.45- μ m olefin polymer (OP) syringe-tip filters. Then, phenolic acids and flavonoids present in the extracts were identified by matching the retention time against their corresponding standard. In this study, the standards used for comparison were gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, isovitexin and quercetin-3- β -D-glucoside (Figure 1). Quantitative analysis was made according to the linear calibration curves of standards compounds. Three replications were made at least for each standard and plant extract.

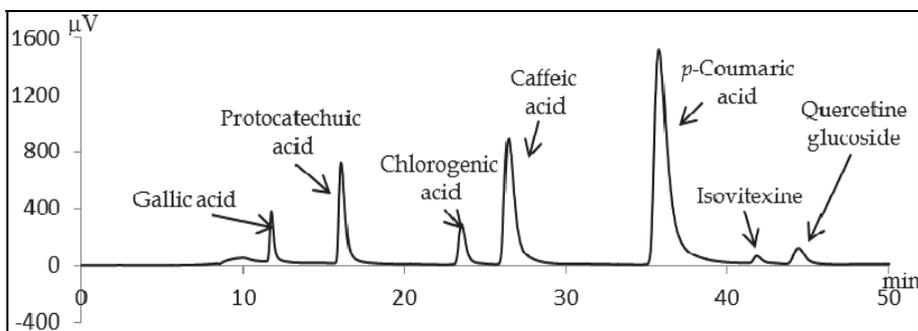


Fig. 1. Chromatogram of standards (1mg/ml)

3. Results

3.1 Total phenolics and total flavonoids

The results of the colorimetric analysis of total phenolics expressed as Gallic Acid Equivalents (GAE) and those of total flavonoids expressed as Catechine Equivalents (CE) are given in the table 2.

Plants name	Plant parts	Total phenolics (mg GAE)	Total Flavonoids (mg CE)
<i>A. leiocarpus</i>	L	223.1 ± 0.2	38.9 ± 1.7
	TB	26.5 ± 0.4	10.3 ± 0.3
<i>C. populnea</i>	RB	76.4 ± 1.1	27.6 ± 1.2
<i>M. inermis</i>	TB	19.5 ± 0.7	11.1 ± 1.3
<i>T. macroptera</i>	TB	48.5 ± 1.3	14.2 ± 1.4
	RB	219.6 ± 0.4	33.1 ± 1.3
<i>V. heterophylla</i>	L	51.5 ± 0.5	9.3 ± 0.9
<i>Z. mucronata</i>	L	52.2 ± 0.5	14.4 ± 0.8
	RB	19.3 ± 0.6	9 ± 1.6

L= leaves; TB= trunk barks; RB= root bark. Total phenolics expressed as gallic acid equivalent (GAE), total flavonoids expressed as catechin equivalent (CE).

Values are means of triplicate determination ± standard deviation.

The total phenolic compounds which are present in plant materials were ranged from 19.3 ± 0.6 to 223.1 ± 0.2 mg GAE / g dry weight and the total amount of flavonoids varied from 9 ± 1.6 to 38.9 ± 1.7 mg CE / g dry weight.

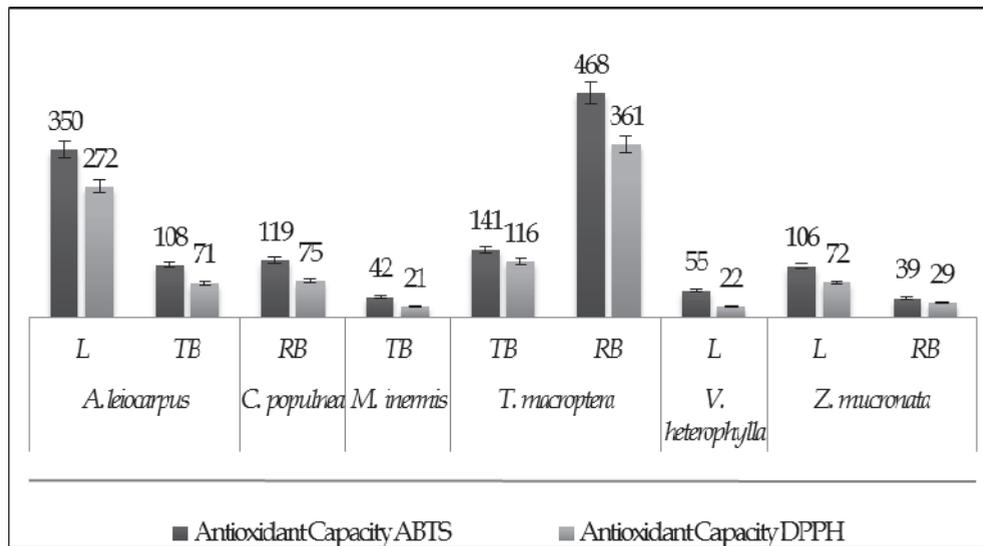
Table 2. Total phenolic and total flavonoid contents of the plant parts

3.2 ABTS and DPPH radical-scavenging activity

ABTS and DPPH tests were conducted to evaluate the antioxidant properties of plant part extract on their stable free radicals in comparison to the antioxidant activity of vitamin C, the corresponding results were collected in the figure 2.

The antioxidant activity using ABTS varied from 39 mg to 468 mg VCE per g dry weight. The overall antioxidant capacity of plant parts in VCEAC, which was evaluated by ABTS assay, was in the following order: *T. macroptera* root bark > *A. leiocarpus* leaves > *T. macroptera* trunk bark > *C. populnea* root bark > *A. leiocarpus* trunk bark > *Z. mucronata* leaves > *V. heterophylla* leaves > *M. inermis* trunk bark > *Z. mucronata* root bark.

The antioxidant activity using DPPH ranged from 21 mg to 361 mg VCE per g dry weight. The overall antioxidant capacity of plant parts in VCEAC which was evaluated by DPPH assay decreased in the following order: *T. macroptera* root bark > *A. leiocarpus* leaves > *T. macroptera* trunk bark > *C. populnea* root bark > *Z. mucronata* leaves ≈ *A. leiocarpus* trunk bark > *Z. mucronata* root bark > *V. heterophylla* leaves > *M. inermis* trunk bark.



L= leaves; TB= trunk barks; RB= root barks

Values are means of triplicate determination ± standard deviation.

Fig. 2. Vitamin C equivalent antioxidant capacity (VCE mg/g dry weight).

3.3 Analysis of polyphenolic composition in plant part extracts using HPLC

The RP-HPLC results which are summarised in table 3, show that the protocatechuic acid (792.00 - 7.93 mg / 100g dry weight), the *p*-coumaric acid (1833.56 - 4.2 mg / 100g dry weight), the gallic acid (69.00 - 5.14 mg / 100g dry weight) and the chlorogenic acid (2286.08 - 62.09 mg / 100g dry weight) were encountered in high concentration, whereas the caffeic acid (149.86 -43.46 mg / 100g dry weight), the isovitexin (182.22 - 31.46 mg / 100g dry weight) and the quercetin-3-β-D-glucoside (83.53 - 70.89 mg /100g dry weight) were found in low concentration. It should be noted that the trunk and the root barks of *T macroptera* and the leaves of *V. heterophylla* contain the greatest number of compounds similar to the standards, whereas none of the standards was detected for the root barks of *Z. mucronata*.

4. Discussion

The leaves of *A. leiocarpus* had the highest total phenolic contents, which was 4- fold higher than those of the leaves of *V. heterophylla*. The lowest total phenolic and total flavonoid contents were found in the leaves of *V. heterophylla*. In the trunk barks of *T. macroptera*, the total phenolics and the total flavonoids were ranked first, followed by *A. leiocarpus* and then the *M. inermis* ones. The phenolic and flavonoid compounds were found in highest concentration first in the root barks of *T. macroptera* followed by the root barks of *C. populnea*. In contrast, it appeared that the lowest amount of flavonoids was found for the root barks of *Z. mucronata*. The total phenolic and the total flavonoid contents of the root barks of *T. macroptera* were respectively 11-fold and 4-fold greater than those of *Z. mucronata*.

Plant	Plant parts	Gallic Acid	Protocatechuic Acid	Chlorogenic Acid	Caffeic Acid	<i>p</i> -coumaric Acid	Isovitexin	Quercetin
<i>Anogeissus leiocarpus</i>	L	49.1 ± 3.5	67.6 ± 0.3	2286 ± 80	nd	nd	nd	nd
	T B	5.1 ± 0.2	35.5 ± 0.2	nd	nd	221 ± 20	nd	nd
<i>Cissus populnea</i>	R B	nd	26 ± 1.2	62.1 ± 0.5	nd	4.2 ± 0.2	nd	70.9 ± 0.5
<i>Mitragyna inermis</i>	T B	5.9 ± 0.16	53.2 ± 1.2	nd	nd	7.5 ± 0.6	nd	nd
<i>Terminalia macroptera</i>	T B	16.4 ± 2.2	7.9 ± 1.3	67 ± 2	nd	59.3 ± 2.2	182 ± 42	nd
	R B	69 ± 1.4	792 ± 7	113.4 ± 1.4	149.9 ± 2.6	1833 ± 4.6	nd	nd
<i>Vepris heterophylla</i>	L	7.5 ± 0.44	10.1 ± 0.7	79 ± 1.7	nd	31.9 ± 2.15	31.5 ± 1.1	nd
<i>Zizyphus mucronata</i>	L	nd	53 ± 0.4	683 ± 20.1	43.5 ± 1.4	nd	nd	nd

L= leaves; TB= trunk barks; RB= root barks; nd= not detected.

Table 3. Concentrations of flavonoids and phenolic acids in the medicinal traditional plant parts (mg / 100g of dry material)

The results obtained by ABTS and DPPH tests show that the antioxidant activity order for these different plant parts was approximately similar in both assays. However, the antioxidant capacity using DPPH compared to the one obtained by ABTS assay was underestimated about 33%. Arnao, (2000) and Delgado-Andrade et al., (2005) report the same occurrence and they explain that the DPPH is only dissolved in alcoholic media. In contrast, the ABTS radicals being solubilised in aqueous and in organic media the antioxidant activity measured is due to the hydrophilic and lipophilic nature of the compounds. In addition, at 515 nm near the visible region where the antioxidant activity is measured, interferences occur with the DPPH coloration.

In this study, we found that phenolic compounds are the major contributors to the antioxidant activity, since total phenolics and antioxidant activity showed a good correlation with a correlation coefficient of $R^2=0.9208$. However, we note that the trunk barks of *A. leiocarpus* exhibit a high antioxidant activity and a low level of total phenol antioxidant. The value of correlation coefficient between total flavonoids and antioxidant activity was $R^2 = 0.752$ only.

These results showing good antioxidant activity of these plant parts are particularly interesting since the antioxidant agents would induce analgesic, anticarcinogenic, anti-

inflammatory, antithrombotic, immune modulating and anti-atherogenic effects (Djeridane et al., 2006).

The results of HPLC analysis were in accordance with those previously reported in the literature. The phytochemical investigations of the different parts of *T. macroptera* led to the isolation of several C- and O-glycosyl flavones, chlorogenic acid, quercetin, gallic acid (Silva et al., 2000). Chyau et al., (2006) identified 3,4-dihydroxybenzoic acid (protocatechuic acid), *p*-coumaric acid, gallic acid from the leaves of *T. catappa*. Moreover, gallic acid was also present in the trunk barks of *A. latifolia* (Govindarajan et al., 2004). Protocatechuic acid (3,4-dihydroxybenzoic acid) was found in *Mitragyna rotundifolia* (Kang & Hao, 2006). Ojekale et al., (2006) have reported the presence of flavonoids in *C. populnea*. In this study, the presence of isovitexin in the leaves of *V. heterophylla* was identified and quantified.

5. Conclusion

This study permits to evaluate the amount of phenolics, flavonoids and their total antioxidant activity linked to six traditional medicinal plants. Antioxidant activity varied greatly among the different plant parts and was highly correlated with the polyphenolic contents. We take an interest in the leaves of *A. Leocarpus* and in the root barks of *T. Macroptera*, since they exhibited important antioxidant activities and could be attractive sources of natural antioxidants. Moreover, this comparative study permits to identify and determine by RP-HPLC, five individual phenolic acids and two flavonoids that are mainly at the origin of the antioxidant activity in the studied plant parts.

6. Acknowledgement

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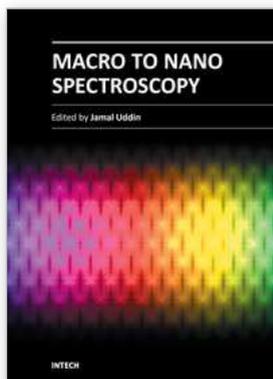
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