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Evaluation of Antioxidant Activity, Total Phenolic and Flavonoid Contents of *Entada africana* Guill. et Perr. (Mimosaceae) Organ Extracts

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Abstract: *Entada africana* is an endangered West African medicinal plant because of a large scale use of its roots. To evaluate and compare the medicinal potential of all plant parts for a sustainable use the radical scavenging activity (DPPH method) and the phenolic compound contents (Folin-Ciocalteu method) of aerial parts and roots were investigated. Aqueous extracts of leaves, stem barks and roots were used for this investigation. Methanol extract was also used for the roots. The fourth extracts showed high radical scavenging activity (ranging from 1.27-3.36 μg mL^{-1}) similar to those of quercetin, rutoside and ascorbic acid (0.8, 5.20 and 5.32 μg mL^{-1}, respectively). Fractions obtained from the four extracts with chloroform, ethyl acetate, methanol and water also showed high activity with EC_{50} ranging from 3.39-16.72 μg mL^{-1} except chloroform fractions (EC_{50}>36 μg mL^{-1}). The total phenolic and flavonoid contents of the four extracts and their twelve fractions were quite high, ranging from 17.10-43.50% TAE. The leave extracts showed an interesting free radical scavenging activity and phenolic content as to be preconized for use instead of the roots.

Key words: *Entada africana*, antioxidant activity, total phenolics, flavonoids

INTRODUCTION

Excess of free radicals that naturally occur in mammalian body through oxidative process is known to be involved in many human diseases such as Alzheimer, ageing process, cataracts, acute liver toxicity, cardiovascular diseases, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, inflammatory process and DNA damage that can lead to carcinogenesis (Favier, 2003; Kassab *et al*., 2003; Atawodi, 2005). The level of these species produced by mitochondrial respiration, phagocytosis, redox cycles or radiation is maintained by neutralizing excess free radical species by nutritional trappers (vitamins C, E, carotenoids, polyphenols) or destruction by various enzyme systems (superoxide dismutases, glutathione peroxidases). Unfortunately, oxidative stress can result from a disruption of the balance between the systems generating free radicals and systems permitting their elimination leading to excess of free radicals that are highly reactive oxygen species; superoxide ($O^{\bullet}_2$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), peroxinitrite ($^{\bullet}ONOO$) and nitric oxide ($NO^{\bullet}$) (Atta-Ur-Rahman and Choudhary, 2001). For the prevention and treatment of these diseases involving for the treatment of these diseases, whose mechanisms involve the process of oxidative stress, many antioxidant based drug formulations are used (Wong *et al*., 2006). Because of restrictions on synthetic antioxidants due to their carcinogenicity, interest has increased considerably in finding naturally occurring antioxidants for use in foods, cosmetics or medicine materials to replace the synthetics ones (Sasaki *et al*., 2002).

Indeed, phenolic compounds found in vegetables, fruits or medicinal plants are known for their antioxidant potential and their role in prevention of human diseases (Cai *et al*., 2004) and number of papers highlighted a positive correlation between the antioxidant activity and the total phenolic content (Tawaha *et al*., 2007). *Entada africana* is a well known leguminous tree used in traditional medicine throughout West-Africa in the

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treatment of many diseases such as fever, respiratory tract complaints, diabetes, hypertension, diarrheas (Nacoulma, 1996; Occhiuto *et al*., 1999). Many studies reported the wound-healing, haemostatic, anti-rheumatism, anti-inflammatory and hepatoprotective properties of this species extracts (Oliver-Bever, 1986; Burkill, 1997; Diallo *et al*., 2001). Traditionally, the roots bark are the most used part of the plants in medicine. Because of this non sustainable root uses, the availability of the plant in Burkina Faso Plateau Central Region is endangered.

The previous research (Tibiri *et al*., 2007) indicated a strong antioxidant activity which could explain the biological activities. The results also indicated that phenolic compounds were an important component of the extracts. Most of these researches were on the organic solvent extracts. To evaluate the plant aerial parts (particularly the leaves) as an alternative to the roots and stem barks, it is necessary to know the biological activity and phenolic contents of all parts of the plant. This research also uses the aqueous extracts as do the traditional healers.

MATERIALS AND METHODS

Plant material: Roots, stem barks and leaves of *Entada africana* were collected in Gampela (Burkina Faso) in September 2009. They were kindly identified by Pr. Millogo, botanist and a voucher specimen was deposited under the registration number TA05-1 in the herbarium of the University of Ouagadougou. The different parts of the plant collected were separately dried under ventilation at room temperature then finely ground with an electrical grinder.

Solvent and reagents: All the solvents, reagents and standards were purchased from Sigma Aldrich (France). The solvents and reagents used for Thin Layer Chromatography (TLC) were analytical grade. TLC was performed over pre-coated silica plates (GF_{254}, Merck). The Folin-Ciocalteu's Reagent (FCR) for total phenolic compounds determination was freshly prepared according to the method described by Singleton *et al*. (1999).

Extraction and fractionation: Leaves, stem barks and roots (50 g of each) were extracted for 30 min by decoction in water. The extract filtrates were lyophilized. Fifty grams of roots were also extracted by maceration in methanol at room temperature for 24 h. The filtrate of this methanol extract was concentrated under reduced pressure until all the methanol had evaporated. The concentrate was redissolved in distilled water and lyophilized. One gram of each aqueous crude extract lyophilisat was then sequentially extracted with chloroform, ethyl acetate and methanol; the residue was re-dissolved in water. For the methanol crude extract lyophilisat, the fractionation order was chloroform, ethyl acetate, water then methanol. The crude extracts and their fractions were subjected to free radical scavenging activity using DPPH$^{\bullet}$ and the content of total phenolics and flavonoids was measured.

Free radical scavenging activity assessment (DPPH$^{\bullet}$ assay): The antioxidant activity of the crude extracts and fractions was assessed by the mean of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) colorimetric method as described by Velázquez *et al*. (2003), slightly modified. This method depends on the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazine and the remaining DPPH$^{\bullet}$, which showed maximum absorption at 517 nm was measured (spectrophotometer Agilent 8453E). About 2 mL of a 20 mg mL^{-1} DPPH$^{\bullet}$ solution were added to 1 mL of a methanolic solution of each extract (1-100 μg mL^{-1}). A mixture of 2 mL of DPPH$^{\bullet}$ and 1 mL of methanol served as control. The mixture was shaken vigorously then incubated for 15 min in darkness at room temperature. Absorbance was measured at 517 nm. Methanol was used as blank. Rutoside, ascorbic acid and quercetin solutions were used as positive controls. Each experiment was performed in triplicate. The DPPH$^{\bullet}$ radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, A_{sample} and $A_{control}$ are absorbance of sample and control. The concentration of sample required to scavenge 50% of DPPH$^{\bullet}$ (EC_{50}) was determined by linear regression for the dose/effect results. Decreasing of the DPPH$^{\bullet}$ solution absorption indicates an increase of DPPH$^{\bullet}$ radical scavenging activity.

Phytochemical screening and thin layer chromatography: Tests for sterols and triterpernes, phenolic compounds, flavonoids, tannins, carbohydrates/glycosides, saponins and alkaloids were performed according to Ciulei (1982). Thin layer chromatography for the presence of secondary metabolites was carried out using TLC plates (Silica gel plates, GF_{254}, Merck) with different eluting systems (Wagner and Bladt, 1996; Lamien *et al*., 2005). The solvent systems were ($CHCl_3$-$AcOOH_G$-MeOH-H_2O, 64:32:12:8), (AcOEt-MeOH- H_2O, 10:0,5:1), (Toluène-$CHCl_3$-EtOH, 40:40:10), ($CHCl_3$-MeOH-H_2O, 65:25:4), (AcOEt-MeOH-H_2O, 100:13,5:10), (MeOH-$CHCl_3$, 3:7), (AcOEt-HCOOH-

$AcOOH_G$-H_2O, 100:11:11:26), (nHex-AcOEt-$AcOOH_G$, 90:40:10). To develop the spots of chromatograms, different spray reagents were used: 1% ferric chloride (tannins), 2% aluminium chloride in ethanol (flavonoids), 3% sulphuric acid/ethanol (saponins), 5% potassium hydroxide (coumarins, anthracenosic derivatives) and Dragendorff reagent (alkaloids).

Total phenolic content: The total phenolic content of plant extracts was assessed using FCR (Singleton *et al*., 1999). This method depends on the reduction of FCR by phenols to a mixture of blue oxides which have a maximal absorption in the region of 760 nm. The intensity of blue staining produced is proportional to the total quantity of phenolic compounds present in the testing samples. For the preparation of calibration curve, 1 mL aliquots of 0.10-0.60 mg mL^{-1} ethanolic tannic acid solutions were mixed with 1 mL FCR 2N and 3 mL sodium carbonate (20%) then left 40 min at room temperature. The absorbance was read at 760 nm and the calibration curve was drawn. 0.25 mL of each extract solution (0.25 g L^{-1}) was completed to 1 mL with distilled water and then mixed with the same reagents as described above. The resulting solution was vortexed and left for 40 min before measuring absorbance. All determinations were performed in triplicates. Results were expressed as Tannic Acid Equivalent (TAE) and phenolic content as percentage of dry matter was calculated from the following formula:

$$X = \frac{C_{tube} \times D}{C_{initial}} \times 100$$

Where:

X = Total phenolic content
C_{tube} = Phenolic concentration of the solution in test tube (g mL^{-1} TAE)
D = Diluting factor
$C_{initial}$ = Concentration of the stock solution (g mL^{-1})

Flavonoid content: Flavonoids were precipitated by formaldehyde at pH<0.8. Five milliliters of a concentrated HCL sol. (50/50 v/v) and 5 mL of formaldehyde (8 mg L^{-1}, in distilled water) were added to 10 mL of each extract or fraction. The mixture was vortexed, then left 24 h at room temperature. Flavonoids were separated by centrifugation (3000 rmp, 10 min) and the supernatant, containing all phenolic compounds except flavonoids (non-flavonoidic phenolics), was collected and filtrated. Its absorbance was measured in the same way as for the total phenolics. Flavonoids concentration was calculated from the values obtained for the non-flavonidic phenolic compounds. The flavonoid content as percentage of dry matter is (X-Y)%, where X is the total phenolic content and Y the non-flavonoidic phenolic content as calculated.

Statistical analysis: Experimental values are mean±SEM of the number of experiments (indicated in the legends). Data were evaluated for statistical significance with one way ANOVA followed by Dunnett's multiple range tests when appropriate (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA). A p-value of 0.05 or less was considered as statistically significant.

RESULTS AND DISCUSSION

Free radical scavenging activity by DPPH˙: As shown in Table 1, the four crude extracts and their fractions, except chloroform fraction, showed a high radical scavenging activity with EC_{50} values ranging from 1.27 μg mL^{-1} (methanol crude extract of the roots) to 16.72 μg mL^{-1} (methanol fraction of the leaves). With EC_{50} values of 1.27, 1.36 and 1.41 μg mL^{-1}, respectively, crude methanol extract of roots, crude aqueous extract of roots and crude aqueous extract of stem barks showed free radical scavenging activity higher than those of quercetin, rutoside and ascorbic acid (EC_{50} values of 2.52, 5.20 and 5.32, respectively). The ethyl acetate fraction of the stem barks extract and the leaves crude aqueous extract and water fraction shower a radical scavenging activity higher than those of rutoside and ascorbic acid. With a EC_{50} of 36.37 μg mL^{-1}, the chloroform fraction of the stem barks aqueous extract showed a moderate activity, EC_{50}<50 μg mL^{-1}. The other chloroform fractions showed a poor radical scavenging activity, EC50>100 μg mL^{-1}.

Phytochemical screening: The four crude extracts were subjected to phytochemical tests and TLC analysis due to their high radical scavenging activity. This phytochemical analysis revealed (Table 2) the presence of phenolic compounds, tannins, flavonoids, coumarins and anthocyans, as major principle components of the different parts of the plant. Phytochemical screening also revealed that *Entada africana* was rich in terpenic and steroidic components (sterols, triterpernes and saponins).

Total phenolics and flavonoids: The result of phytochemical screening that showed that phenolic compounds constitute one of the major components of the extracts led to the determination of total phenolics and flavonoids contents of the different extracts and active fractions. As shown in Table 3, the crude extracts (water and methanol) and their fractions have high

Table 1: Extraction yield and EC_{50} values against DPPH' of *Entada africana* aqueous extracts

	Crude extracts and fractions									
	Water (crude extract)		Chloroform		Ethyle acetate		Methanol		Water	
Parts of plants	Yield (%)	EC_{50} (μg mL^{-1})	Yield (%)	EC_{50} (μg mL^{-1})	Yield (%)	EC_{50} (μg mL^{-1})	Yield (%)	EC_{50} (μg mL^{-1})	Yield (%)	EC_{50} (μg mL^{-1})
Leaves	6.40	3.36±0.02[b]	2.12	344.20±8.99	8.18	9.31±0.09	47.94	16.72±0.18	32.00	3.39±0.11[b]
Barks	6.66	1.36±0.03[a,b]	0.06	36.37±2.79	1.64	8.71±0.17	42.34	7.56±0.21	31.50	11.74±0.05
Roots	4.80	1.41±0.01[a,b]	0.76	499.50±16.66	4.96	4.92±0.10[b]	35.40	10.51±0.10	25.50	13.90±0.14
Roots (Methanol)	9.64	1.27±0.01[a,b]	11.50	102.20±1.00	18.32	7.39±0.20	22.22	8.10±0.18	29.26	6.60±0.08

Quercetin, 0.87±0.06, Rutoside, 5.20±0.14, Ascorbic acid, 5.32±0.12. Values are the means ± SEM (n = 3). Data were analyzed by ANOVA ([a]$p<0.05$ compared to quercetin, [b]$p<0.05$ compared to quercetin, rutoside and ascorbic acid

Table 2: Results of phytochemical screening of roots, stem barks and leaves of *Entada africana*

Chemical groups	Stem barks	Roots	Leaves
Phenolic compounds			
Coumarins and derivatives	+++	++	+++
Flavonic aglycones	-	-	-
Anthracenosids	-	++	-
Flavonoids	+++	+++	+++
Gallic tannins	+++	+++	+++
Catechic tannins	-	-	-
Anthocyanosids	+++	+-	+++
Terpenic and steroidic compounds			
Carotenoids	+	-	+++
Sterols et triterpernes	+++	+++	++
Steroidic and triterpenic heterosids	+++	++	+++
Saponosids	+++	+++	+++
Cardiotonics	-	-	-
Others			
Alkaloids	-	-	-
Reducing compounds	+++	+++	+++
Emodols	++	-	-

- Absent, + Present, ++ Abundant, +++ Very abundant, ± Traces

Table 3: Total phenolic content of aqueous extracts of leaves, stem barks and roots of *Entada africana,* expressed in TAE (Tannic Acid Equivalent)

	Total phenolic content							
	Water (crud extract)		Ethyl acetate		Methanol		Water	
Parts of plants	μg mL^{-1}	%	μg mL^{-1}	%	μg mL^{-1}	%	μg mL^{-1}	%
Leaves	2.55±0.08	20.39	3.81±0.23	30.46	2.87±0.06	22.98	2.33±0.33	18.67
Barks	3.74±0.07	29.91	3.24±0.25	25.88	2.97±0.28	23.78	4.08±0.51	32.66
Roots	2.14±0.05	17.10	3.98±0.23	31.84	2.76±0.06	22.08	3.07±0.03	24.55
Roots (Methanol)*	4.13±0.03	33.02	4.58±0.21	36.61	5.44±0.07	43.50	3.82±0.08	30.52

Values are the means±SEM (n = 3). *Methanol crude extract of the roots

content in total phenolics, ranging from 17-43%. Methanol fraction of methanol crude extract of the root has the highest total phenolic content, up to 43%. The ethyl acetate, methanol and water fractions of the methanol crude extract (roots) showed the highest content in flavonoids, up to 15.78% in the ethyl acetate fraction (Table 4). The flavonoid content of methanol fraction of the aqueous crude extracts was weak (<1%).

DPPH method is commonly used to assess radical scavenging of any antioxidant substance because it is a quick, reliable and reproducible method to search *in vitro* general antioxidant of pure compounds as well as plant extracts (Koleva *et al*., 2002). The methanol crude extract of the roots and the aqueous crude extracts of roots, stem barks and leaves of *Entada africana* as well as their fractions in ethyl acetate, methanol and water showed a high radical scavenging activity. This activity was comparable to those of rutoside and ascorbic acid, well known antioxidant compounds with health benefit for human (Hollman *et al*., 1996; Gordon and Roedig-Penman, 1998; Morales *et al*., 2006; Ameho *et al*., 2008; Boots *et al*., 2008). The high antioxidant activity of *Entada africana* supports its frequent use in traditional medicine for the treatment of many diseases including wounds, malaria, hepatic diseases and diabetes. These results are consistent with those obtained by Cook *et al*. (1998) with the Trolox assay on the leaves and those of Tibiri *et al*. (2007) on free radical scavenging activity with

Table 4: Flavonoids content of aqueous extracts of leaves, stem barks and roots of *Entada africana*, expressed in TAE (Tannic Acid Equivalent)

	Crude extracts and fractions							
	Water (crud extract)		Ethyle acetate		Methanol		Water	
Contents of aqucous extracts	µg mL^{-1}	%	µg mL^{-1}	%	µg mL^{-1}	%	µg mL^{-1}	%
Non-flavonoidic phenolics								
Leaves	2.40±0.15	19.16	3.44±0.15	27.53	2.84±0.06	22.70	1.43±0.26	11.44
Barks	2.36±0.12	18.85	3.00±0.10	23.99	2.86±0.01	22.90	3.21±0.20	25.70
Roots	1.00±0.08	8.00	3.56±0.39	28.45	2.73±0.10	21.82	0.68±0.05	5.44
Roots (Methanol)	2.95±0.02	23.61	2.60±0.19	20.82	4.35±0.07	34.78	2.22±0.04	17.76
Flavonoids*								
Leaves	-	1.23	-	2.93	-	0.27	-	7.23
Barks	-	11.06	-	1.89	-	0.87	-	6.95
Roots	-	9.10	-	3.39	-	0.26	-	19.11
Roots (Methanol)**	-	9.41	-	15.78	-	8.71	-	12.76

Values are the means±SEM (n = 3); *Calculated from the values of non-flavonoidic phenolics. **Methanol crude extract of the roots

methanol extracts of the leaves and barks. With a scavenging activity superior to that of rutoside and ascorbic acid, the present study seems to indicate that the crude aqueous extracts of the different parts of the plant and the crude methanol extract of the roots have higher antioxidant activity than the methanol extracts of the aerial parts as previously obtained by Tibiri *et al.* (2007). Indeed, the highest antioxidant activity against DPPH$^{\bullet}$ obtained with methanol extracts of the aerial parts was merely in the same order that those of rutoside and ascorbic acid. The high content in phenolic compounds of the extracts as showed by the total phenolic and flavonoid content assessment is consistent with the free radical scavenging activity obtained.

Number of papers report a strong correlation between antioxidant activities of plants extracts and their phenolic compound content (Almela *et al*., 2006; Abdel-Hameed, 2009; Arcan and Yemenicioglu, 2009), specifically, flavonoids have a good reputation for their antioxidant properties (Rice-Evans *et al*., 1996; Choi *et al*., 2002; Firuzi *et al*., 2005; Mariani *et al*., 2008).

The phytochemical screening also showed the abundance of terpenic and steroidic compounds in *Entada africana*. These results show a similarity in chemical composition between *Entada africana* and other species of the genus as *Entada abyssinica* and *Entada phaseoloides*.

Indeed, diterpene derivatives and saponins have been isolated from these plants (Okada *et al*., 1987; Barua *et al*., 1988; Freiburghaus *et al*., 1998; Nyasse *et al*., 2004). Nine new triterpenic saponins actives against some tumor cells have been isolated from the roots of *Entada africana* (Cioffi *et al*., 2006).

The terpenic and steroidic compounds are known for their anti-inflammatory and hepatoprotective properties (Liu, 1995), their abundance in the plant could explain its use in traditional medicine against hepatitis and malaria fever and as wound-healing.

CONCLUSION

With the growing knowledge of the mechanisms of human diseases, particularly metabolic diseases like diabetes, liver diseases and hypertension, the role played by highly reactive oxygen species such as free radicals becomes increasingly relevant. Research on medicinal plants for natural antioxidants is also increasing. The strong free radical scavenging activity of *Entada africana* shown in this research and in previous research encourages further studies for the isolation and identification of active compounds. In addition, this study, complementing the previous one, shows that the aerial parts of the plant also have therapeutic potential, which may be an alternative to the use of its roots.

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