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Polyphenol Contents and Antioxidant Activities of Five *Indigofera* Species (Fabaceae) from Burkina Faso

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Abstract: Aqueous acetone extracts prepared from five *Indigofera* species of Burkina Faso, namely *Indigofera colutea* (Burm.) Murril., *I. macrocalyx* Guilld et Perr., *I. nigritana* Hook f., *I. pulchra* willd. and *I. tinctoria* L., were investigated for their phytochemical composition and their antioxidant activities. Standard methods and TLC were used to screen the phytochemical composition. The total phenolic and flavonoid content of extracts were assessed by Folin-Ciocalteu and $AlCl_3$ methods, respectively. These extracts were also evaluated for their antioxidant potentials using ferric reducing antioxidant power (FRAP), 2,2-diphenyl-l-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) assays. Flavonoids, saponins, quinones, sterols/triterpenes and tannins were present in all these species except for *I. pulchra* where quinones were not found. Gallic acid, caffeic acid, rutin and myricetin in *I. colutea*; gallic acid, quercitrin, myricetin in *I. tinctoria*; galangin and myricetin in *I. macrocalyx* were identified by thin layer chromatography. Among these, *I. colutea*, *I. tinctoria*, *I. nigritana* and *I. macrocalyx*, which had the highest phenolic content, were also found to possess the best antioxidant activities. The results indicated a good correlation between antioxidant activities and total phenolic content ($p<0.05$ for FRAP/DPPH and DPPH/ABTS and $p<0.01$ for FRAP/ABTS). These plants represent promising sources of natural antioxidants and these findings give scientific bases to their ethnopharmacological uses.

Key words: Antioxidant, phenolic, flavonoid, *Indigofera*, Burkina Faso

INTRODUCTION

The genus *Indigofera* comprises around 700 species that are distributed geographically in tropical regions. In Burkina Faso, Nigeria and India, *Indigofera colutea* (Burm.) Murril., *I. macrocalyx* Guilld et Perr., *I. nigritana* Hook f., *I. pulchra* willd. and *I. tinctoria* L. have intensive popular use in the treatment of malaria, dysentery, constipation, stomach ache, fatigue, skin disease and wounds (Table 1).

Rotenoids isolated from *I. tintoria* were found to be toxic to larvae of *Anopheles stephensi* and adults of *Callosobruchus chinensis* (Kamal and Mangla, 1993).

Table 1: Medicinal uses of *Indigofera* species

Plants	Part used	Medicinal uses
I. colutea	Whole plant	Stomach ache (Perumal Samy *et al*., 1998)
I. macrocalyx	Inflorescence, Stems, leaves	Pyrgative, Malaria, delay of children's locomotion, sexual impotence, constipation, stimulant (Nacoulma, 1996; Kerharo and Adams, 1974)
I. nigritana	Stems, leaves	Malaria, children gastro-intestinal pain, skin disease, fatigue, antifungal, antiseptic, stimulant, dysentery (Nacoulma, 1996)
I. pulchra	Whole plant	Snake-bites (Abubakar *et al*., 2006)
I. tinctoria	Whole plant	Febrifuge, antipyretic, wounds, antiseptic, Diuretic fatigue, sexual impotence, snake-bites, sexual infections, constipation, pain (Nacoulma, 1996)

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Recently, antidyslipidemic activity (Narender *et al*., 2006) and hepatoprotective effects (Singh *et al*., 2006) of *I. tinctoria* have been reported. Except these data, little is known on the biological properties of *Indigofera* species despite their intensive uses by local inhabitants in West Africa and India. Therefore, investigation on the biological properties of *Indigofera* species is needed for their safe and efficient use.

Among the biological potential of medicinal plants, the antioxidant activity has gained an increase interest these last years because of the role that they play in the prevention of chronic ailments such as heart disease, cancer, diabetes, hypertension, stroke and Alzheimer's disease by combating oxidative stress (Cole *et al*., 2005; Liu, 2003; Riboli and Norat, 2003).

According to some researchers, vitamins, phenolic and carotenoids are the major natural antioxidant groups (Rice-Evans *et al*., 1997; Gülçin, 2006; Thaipong *et al*., 2006). Phenolic compounds are secondary metabolites widespread in the plant kingdom and which differ widely in terms of structure and biological properties.

The aim of the present study was to examine the total phenolic and flavonoid contents as well as the antioxidant activity of five *Indigofera* aqueous acetone extracts. The antioxidant potential has been determined using ferric reducing antioxidant power assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) free radical scavenging assays.

MATERIALS AND METHODS

Chemicals: The Folin-Ciocalteu reagent, NaH_2PO_4, Na_2HPO_4, sodium carbonate, aluminium trichloride ($AlCl_3$), diphenylboric acid 2-aminoethyl Ester (diphenyl-boryloxyethylamine), gallic acid and quercetin were purchased from Sigma aldrich chemie, Steinheim, Germany. 2,2-diphenyl-l-picrylhydrazyl (DPPH); 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) ABTS, polyethylene glycol 4000, trichloroacetic acid, potassium persulfate and solvents used were from Fluka Chemie, Buchs, Switzerland. Potassium hexacyanoferrate [$K_3Fe(CN)_6$] was from Prolabo, Paris, France and ascorbic acid was from Labosi, Paris, France. All chemicals used were of analytical grade.

Plant material: *Indigofera colutea* (Burm.) Murril., *I. macrocalyx* Guilld et Perr., *I. nigritana* Hook f., *I. pulchra* willd. and *I. tinctoria* L. were collected in the Ouagadougou region of Burkina Faso in August 2005 and identified by Pr. J. Millogo, a botanist from the University of Ouagadougou. Voucher specimen numbers 01, 02, 03, 04 and 05, (respectively for *I. colutea*, *I. marcrocalyx*, *I. nigritana*, *I. pulchra* and *I. tinctoria*) were deposited in the Herbarium of Laboratoire de Biologie et d'Ecologie Végétales UFR/SVT University of Ouagadougou.

Preparation of plant extracts: For each plant, the freshly cut stems with leaves were dried at room temperature and ground to fine powder using a grinder. The extraction was processed using 50 g of powder in 500 mL of acetone:water (80:20) during 48 h under mechanical agitation (SM 25, Edmund BÜHLER, Germany, shaker), at room temperature. After filtration, acetone was removed under reduced pressure in a rotary evaporator (BÜCHI Rotavapor R-200, Switzerland) and the remaining aqueous solutions were lyophilised using a freeze drying system (Cryodos 50, TELSTAR, Spain).

Phytochemical screening and thin-layer chromatography (TLC): The aqueous acetone extract obtained from each plant was used to screen alkaloids, tannins, anthraquinones, flavonoids, saponins, triterpenoids, steroids and coumarins using the method described by Ciulei (1982). The thin layer chromatography analysis of phenolic acids and flavonoids were performed according to Medié-Sarié *et al*. (2004) and Wagner and Bladts (1996) methods.

The thin layer chromatography plates (Silica gel $60F_{254}$, Kiesel gel, 10×10 cm) were spotted with standards or plant extracts and developed in solvent system S1 (Ethyl acetate:formic acid:glacial acetic acid:water, 100:11:11:26) or S2 (n-hexane:ethyl acetate:acetic acid, 62:24:10). Phenolic acids and flavonoids were revealed by spraying the plate with NEU-reagent (Natural products-polyethylene glycol reagent).

Determination of total phenolic and total flavonoid contents: Total phenolic content of each plant extract was determined by Folin-Ciocalteu method (Singleton *et al*., 1999). The diluted aqueous solution of each extract (0.5 mL) at a concentration of 100 μg mL^{-1} was mixed with 2.5 mL of Folin Ciocalteu reagent (0.2 N). This mixture was allowed to stand at room temperature for 5 min and then, 2 mL of sodium carbonate solution (75 g L^{-1} in water) was added. After 2 h of incubation, the absorbencies were measured at 760 nm against a water blank using a spectrophotometer (CECIL CE 2041, CECIL Instruments, England). The standard calibration curve was plotted using gallic acid (0-200 mg L^{-1}). The determination was performed in triplicate and the results were expressed as mg of Gallic Acid Equivalents (GAE)/100 mg of extract.

The total flavonoid content of the plant extract was estimated according to Dowd method, as adapted by

Arvouet-Grand *et al.* (1994). For each extract, 2 mL of methanolic solution (100 μg mL^{-1}) was mixed with 2 mL of aluminium trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415 nm after 10 min against a blank sample consisting of a 2 mL of methanol and 2 mL of plant extract without $AlCl_3$.

Quercetin was used as reference compound to produce the standard curve and the average of three readings was used and expressed as mg of Quercetin Equivalents (QE)/100 mg of plant extract.

Antioxidant activity

Iron (III) to iron (II) reduction activity (FRAP): The total antioxidant capacity of plant extract was determined using iron (III) reduction method (Hinneburg *et al.*, 2006). The diluted aqueous solution of each extract (1 mL) at a concentration of 100 μg mL^{-1} was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of a 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min incubation at 50°C, 2.5 mL of trichloroacetic acid (10 %) was added and the mixture was centrifuged at 3000 rpm for 10 min. Then, 2.5 mL of the upper layer solution was mixed with 2.5 mL of water and 0.5 mL of an aqueous $FeCl_3$ (0.1%) solution. Absorbencies were read at 700 nm and ascorbic acid was used to produce the calibration curve. The iron (III) reducing activity determination was performed in triplicate and expressed in mmol Ascorbic Acid Equivalents per gram of extract.

DPPH radical method: The ability of the extract to scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was determined according to the method of Velazquez *et al.* (2003) with some modifications. Briefly, 1.5 mL of a freshly prepared methanolic solution of DPPH (20 mg L^{-1}) was mixed with 0.75 mL of extract solution (0.003-1 mg mL^{-1}). After 15 min of incubation in the dark, at room temperature, absorbencies were read at 517 nm against a blank sample consisting of a 1.5 mL of methanol and 0.75 mL of extract solution.

Quercetin, ascorbic acid and gallic acid were used as positive controls. All determinations were performed in triplicate. DPPH radical inhibition percentage was calculated according to the formula of Miliauskas *et al.* (2004):

$$\text{Inhibition (\%)} = [(A_B - A_A)/A_B] \times 100$$

where, A_B is the blank absorbance and A_A the sample absorbance (tested extract solution), IC_{50} value was obtained by graphically determination. A lower IC_{50} value indicates greater antioxidant activity.

ABTS radical cation decolorization assay: The radical scavenging capacity of antioxidants for ABTS radical cation was carried out as described by Re *et al.* (1999). The $ABTS^+$ was generated by reacting 7 mM aqueous solution of ABTS with 2.5 mM potassium persulfate (final concentration) followed by storage in the dark, at room temperature, for 12 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70±0.02 unit at 734 using spectrophotometer.

For each extract, a 20 mg mL^{-1} solution in methanol was prepared and further 100 fold diluted in ethanol. Ten microliter of this diluted sample was allowed to react with 990 μL of fresh $ABTS^+$ solution and then absorbance was taken 6 min exactly after initial mixing. Ascorbic acid was used as standard and the capacity of free radical scavenging was expressed as mmol ascorbic acid equivalents g^{-1} of extract.

Statistical analysis: For statistical analysis, MS Excel software (CORREL Statistical function) was used to calculate quercetin, ascorbic acid and gallic acid equivalents, to determine inhibition percentage and to establish linear regression equations. Pearson Product Moment function and One way ANOVA (Tukey test) of SigmaStat 2.0 (Jandel Scientific software) were used to determine correlation coefficients (R) and the level of statistical significance, respectively.

RESULTS AND DISCUSSION

Different *Indigofera* species from Burkina Faso folk medicine have been studies, in order to support and confirm their uses (Table 1).

Firstly, aqueous acetone extracts prepared from studied plants were screened for their phytochemical composition. The dry residues obtained were 5.57 g for *I. colutea*, 6.02 g for *I. macrocalyx*, 5.96 g for *I. nigritana*, 5.28 g for *I. pulchra* and 12.63 g for *I. tinctoria*.

Flavonoids, saponins, quinones, sterols/triterpenes and tannins were detected in all these species with an exception being the absence of quinones in *I. pulchra* (Table 2). The presence of saponin, flavonoid and tannin in some *Indigofera* species such as *I. arrecta*, *I. aspalathoides* and *I. dendroides* has already been reported (Christina *et al.*, 2003; Esimone *et al.*, 1999; Nacoulma, 1996).

Contrasting with the findings of a previous study on a close related species (Leite *et al.*, 2006), namely *I. suffruticosa*, alkaloids and coumarins were not detected in the plants used in this study.

Further confirmation for the presence of phenolic compounds was made by thin layer chromatography.

Table 2: Phytochemical screening of acetone extract of *Indigofera* species

Tested material	Extraction yield (%)	Positive tests for	Negative tests for	Compounds identified by TLC
I. colutea	11.14	Flavonoids, saponins, quinones, sterols/ triterpenes, tannins and/or polyphenols	Alkaloids, Coumarins	Quercitrin, gallic acid, myricetin
I. macrocalyx	12.04	Flavonoids, saponins, quinones, sterols/ triterpenes, tannins and/or polyphenols	Alkaloids, Coumarins	Galangin, myricetin
I. nigritana	11.93	Flavonoids, saponins, quinones, sterols/ triterpenes, tannins and/or polyphenols	Coumarins, Alkaloids	-
I. pulchra	10.56	Flavonoids, saponins, sterols and triterpenes, tannins and/or polyphenols	Coumarins, Quinones, Alkaloids	Rutin
I. tinctora	25.26	Flavonoids, saponins, quinones, sterols/ triterpenes, tannins and/or polyphenols	Coumarins, Alkaloids	Myricetin, gallic acid, rutin, caffeic acid

Table 3: Polyphenol contents and antioxidant activities of *Indigofera* acetone extracts

Plants/references compounds	Total phenolic content (mg GAE/100 mg extract)	Total flavonoids content (mg QE/100 mg extract)	FRAP (ascorbic acid equivalents per gram)	DPPH (IC_{50}: μg mL^{-1})	ABTS (ascorbic acid Equivalents per gram)
I. colutea	54.27±4.87	3.27±1.27^{e}	2.54±0.14	2.45±0.15	3.74±0.14
I. macrocalyx	37.40±0.63^{a}	9.23±0.08^{d}	1.41±0.02^{e}	6.35±0.30	1.70±0.23^{g,h}
I. nigritana	44.20±1.98^{b}	9.63±0.45^{d}	1.57±0.06^{e}	3.68±0.87^{f}	2.24±0.16^{g}
I. pulchra	28.22±0.50	6.83±0.27	0.81±0.01	12.37±0.32	1.36±0.25^{h}
I. tinctoria	42.27±0.55^{a,b}	2.38±0.30^{e}	2.04±0.09	3.79±0.08^{f}	3.00±0.37
Ascorbic acid	-	-		1.80±0.43	-
Gallic acid	-	-	-	0.61±0.14	-
Quercetin	-	-	-	0.88±0.11	-

Results are mean±SD (n = 3), Values with the same letter(s) are not significantly different (p>0.05)

Galangin in *I. macrocalyx* extract, gallic and caffeic acids in *I. tinctoria* and gallic acid in *I. colutea* were identified using the solvent system 2. With the system 1, rutin in *I. pulchra*, rutin and myricetin in *I. tinctoria*, quercitrin and myricetin in *I. pulchra* and myricetin in *I. macrocalyx* were identified (Table 2).

The result shows a qualitative difference in phenolic acids and flavonoids composition between these species. As the detected phenolics have different known biological activities, their presence can partially justify some of the medicinal uses of *Indigofera* species in Burkina Faso. Examples are the anti-inflammatory and antimicrobial activities of galangin, rutin and gallic acid (Bruneton, 1993; Carnat *et al*., 2004; Elliott *et al*., 2000; Rajkapoor *et al*., 2004; Raj Narayana *et al*., 2001; Ueda *et al*., 2002) which can justify the uses of these *Indigofera* species to cure inflammation and skin diseases.

Because the structure of phenolic compounds, mainly the position of the hydroxyl radical, plays a major role in their antioxidant properties (Miliauskas *et al*., 2004), these five *Indigofera* species were studied, keeping in mind the difference in their qualitative composition. For this purpose, the total phenolic and flavonoid content of the different extracts was determined.

Total phenolic and flavonoid content estimated from the calibration curves (Y = 104.83 X, R^2 = 0.9969 for total phenolic and Y= 40.23 X, R^2 = 0.9999 for total flavonoid content) are shown in Table 3. The highest total phenolic content was recorded in *I. colutea* extract (54.27±4.87 mg GAE/100 mg) while the highest amount of flavonoids (9.63±0.45 mg QE/100 mg of dried extract) was found in the extract of *I. nigritana*.

High levels of phenolic were also found in *I. nigritana* and *I. tinctoria* and the lowest flavonoid content which found in *I. colutea* and *I. tinctoria*. These results showed that *I. macrocalyx* and *I. nigritana* have comparable amounts of total phenolic and total flavonoid. No significant correlation was found between the total phenolic and the total flavonoid content.

The FRAP assay determined the reducing power of plant extracts resulting from the ability of their components to donate electrons and, therefore, participate in redox reactions. Using the standard curve of ascorbic acid (Y = 126.9X, R^2 = 0.9999), the best activities were found with *I. colutea* (2.54± 0.14 mmol g^{-1}) and *I. tinctoria* (2.04±0.09 mmol g^{-1}) extract followed by *I. nigritana* (1.57±0.06 mmol g^{-1}) and *I. macrocalyx* (1.41±0.02 mmol g^{-1}) (Table 3). For these four plants, the amounts of ascorbic acid equivalents were significantly higher than that of *I. pulchra* (0.81±0.01 mmol g^{-1}).

The DPPH assay is based on the measurement of the relative inhibition of the extract tested at various concentrations. Chemicals which are able to change the colour of the DPPH free radical from violet to yellow can be considered as antioxidants and therefore, radical scavengers (Hinneburg *et al*., 2006). Table 3 shows that values of the 50% inhibition concentration (IC_{50}) varied from 2.45 0.15 to 12.37 0.32 μg mL^{-1}.

The best antioxidant activity was obtained from *I. colutea* (2.45 ± 0.15 μg mL^{-1}) followed by *I. nigritana* (3.68±0.87 μg mL^{-1}) and *I. tinctoria* (3.79±0.08 μg mL^{-1}), which with the first one, were also found to possess the highest phenolic content. *I. pulchra* with low phenolic content exhibited a relatively weak antioxidant activity.

The IC_{50} values for the references were 1.80±0.43 μg mL^{-1} for ascorbic acid, 0.88±0.11 μg mL^{-1} for quercetin and 0.61±0.14 μg mL^{-1} for gallic acid.

These data show that all these extracts displayed significant antioxidant activity (IC_{50} <15 μg mL^{-1}), but the reference substances were more radical scavengers than the *Indigofera* extracts.

The free radical scavenging ability of *Indigofera* species was also determined using ABTS radical cation. Ascorbic acid was used to produce the dose response curve.

Table 3 shows ascorbic acid equivalents antioxidant capacity of the different extracts in ABTS assay which were estimated from the standard curve of ascorbic acid ($Y = 20.06-33.48X$, $R^2 = 0.9982$).

The strongest antioxidant activities were obtained from *I. colutea* (3.74±0.14 mmol g^{-1}) and *I. tinctoria* (3.00±0.37 mmol g^{-1}) which possess the highest phenolic content. *I. pulchra* with a low phenolic content has shown the weakest antioxidant capacity (1.36±0.25 mmol g^{-1}).

In this study, ascorbic acid has been used as standard instead of trolox, because these two chemicals have almost the same inhibition percentage at 734 nm for ABTS radical cation (Katalinic *et al*., 2006). Several study confirm the usefulness of ascorbic acid (Lee *et al*., 2003; Soong and Barlow, 2004).

In the present study, the highest phenolic content and the best antioxidant activities, using 3 different methods, were recorded in *I. colutea*. These results suggest that it can be a good source of antioxidants.

The antioxidant activities of these different *Indigofera* species can be attributed, in part, to their gallic acid, rutin and myricetin content, due to the strong antioxidant capacity of these chemicals. The high total phenolic content of these plants can also explain, in part, these good antioxidant activities. This last assertion is strongly supported by the correlation studies (Fig. 1) which show good correlations between the total phenolic content and iron reduction ($R = 0.95$, $p<0.05$), DPPH inhibition ($R = 0.99$, $p<0.001$) and ABTS reduction ($R = 0.92$, $p<0.05$). Otherwise, more than 90% of the antioxidant capacity of these plant extracts derives from the contribution of phenolic compounds and the antioxidant activity is not limited to phenolics. The activity may also come from the presence of other antioxidant compounds which, in this case, are contributing for 9% of the antioxidant capacity (Javanmardi *et al*., 2003).

These correlations also suggest that phenolic compounds contribute well to the antioxidant capacity of the *Indigofera* species. Similar correlation were also found

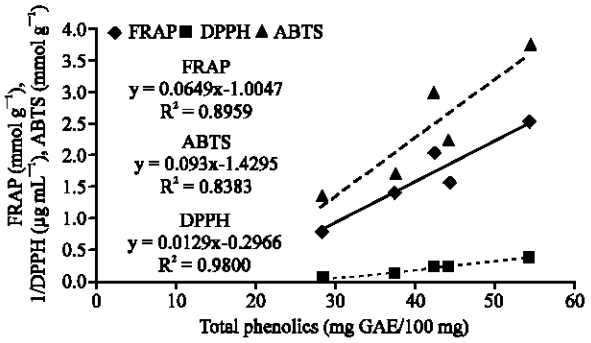

Fig. 1: Correlations between total phenolic content and antioxidants capacities of *Indigofera* extracts

with plant extracts, honey and fruits in earlier study confirming the interest of polyphenols as natural antioxidant from plants and foods (Cai *et al*., 2006; Ivanova *et al*., 2005; Lee *et al*., 2003; Meda *et al*., 2005; Sawadogo *et al*., 2006; Zheng and Wang, 2001). This antioxidant capacity is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Javanmardi *et al*., 2003).

In contrast to the total phenolic content, no significant correlation was found between total flavonoid content and the antioxidant capacities.

A strong correlation have been found between the different antioxidant assays ($R = 0.95$, $p<0.05$ for FRAP/DPPH and DPPH/ABTS and $R = 0.98$, $p<0.01$ for FRAP/ABTS).

The good antioxidant activities of the studied plants can also justify their uses for the treatment of inflammation, as the anti-inflammatory properties of some polyphenols derive mainly from their free radical scavenging activities (Fenglin *et al*., 2004). Nevertheless, further investigations are required to confirm this hypothesis.

This study has shown that *Indigofera* species, used in folk medicine in Burkina Faso, have some phytochemicals with known pharmacological activities. The aqueous acetone extracts of *I. colutea*, *I. tinctoria* and *I. nigritana* which had the highest total phenolic content were found to possess the strongest radical scavengers in both DPPH and ABTS assays and also, the best reduction power in FRAP assay. Good correlations were obtained between DPPH, FRAP and ABTS antioxidant assays and also between each of these assays and the total phenolic content. According to the total phenolic content and antioxidant activities of their extracts, the plants used in this study represent a good source of antioxidant. These results give further support to the therapeutical uses of these *Indigofera* species.

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