

Full Length Research Paper

***In vitro* antioxidant activity and phenolic contents of different fractions of ethanolic extract from *Khaya senegalensis* A. Juss. (Meliaceae) stem barks**

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Received 17 March, 2016; Accepted 25 May, 2016

Khaya senegalensis A. Juss (Meliaceae) is a medicinal plant used in folk medicine of Burkina Faso. Its stem barks are used to treat several diseases such as inflammation, arthritis, infections, ulcer, malaria, fever and dermatosis. The antioxidant activity of aqueous ethanol extract and fractions of *Khaya senegalensis* stem bark was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2'-azino-bis (ABTS•⁺), ferric reducing antioxidant power (FRAP) and lipidic peroxidation methods. Total phenolic, tannins, flavonoids and flavonol contents of extract and fractions were determined. Butanol fraction had the highest value with $IC_{50} = 1.76 \pm 0.19 \mu g ml^{-1}$ (ARP = 0.56) with DPPH• assay, however n-hexan fraction showed the highest capacity to scavenge ABTS•⁺; FRAP values varied from 13.04 ± 0.25 to 13.60 ± 0.09 mmol Trolox Equivalent per gram (mmol TE g⁻¹) of extract or fraction. Ethyl acetate fraction presented the best activity ($70.30 \pm 0.40\%$, $100 \mu g ml^{-1}$) using lipid peroxidation inhibition method. Aqueous fraction contained the highest of total phenolics and tannins contents with, respectively 3.68 ± 0.11 and 2.65 ± 0.18 g TAE/100 g of dry weight (dw) of plant material. Aqueous fraction also showed the highest of total flavonoids (0.04 ± 0.01 g QE/100 g dw) and flavonol (0.10 ± 0.01 g QE/100 g dw) contents. *K. senegalensis* possesses a potential antioxidant effect and contains phenolic compounds. These results provide scientific evidence that validates the use of *K. senegalensis* in traditional medicine.

Key words: *Khaya senegalensis*, antioxidant, phenolic, flavonoids, tannins

INTRODUCTION

Plants play an important role in human life since thousands of years; they provide humanity food, energy (coal and firewood), building material and medicine.

Plants have formed the basis of traditional medicine and provide new remedies through new compounds isolated and used as drugs (Gurib-Fakim, 2006). Secondary

metabolites production by plants are responsible for the therapeutic properties of medicinal plants. Secondary metabolites have been known to be synthesized by plants in response to infectious attack and environmental conditions (Parvin et al., 2015; Ghasemzadeh and Jaafar, 2013). There is a variety of these compounds found in plants such as phenolic compounds that exhibit a wide range of biological properties, including anti-inflammatory, antioxidant, antimicrobial, anticancer, hypoglycemic (Wen et al., 2015).

Many drugs possessing antioxidant property are used to treat oxidative stress. Medicinal plants play a vital role in the production of the antioxidant defense system by providing antioxidant plant phenol (phenolic compounds and flavonoids) (Willcox et al., 2012).

Reactive oxygen species (ROS) and other free radicals produced during metabolism arise from a necessary and normal process that contributes to the defense system of organism. However excessive production of free radical is harmful to the organism, leading to oxidative stress which is associated with the pathogenesis of chronic diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases, arthritis, obesity, and autoimmune disorders (Willcox et al., 2012; Pham-Huy et al., 2008).

Khaya senegalensis A. Juss (Meliaceae) is a medicinal plant used in folk medicine of Burkina Faso. The leaves, stem barks, seeds, and roots of this plant are used to treat several diseases such as inflammation, arthritis, infections, ulcer, malaria, fever, dermatosis. Literature reported that ROS production plays important role in the pathogenesis of inflammation, arthritis, ulcer and malaria (Percário et al., 2012; Mirshafiey and Monireh, 2008). Previous studies had reported the anti-inflammatory, analgesic and antipyretic effects of extracts from stem bark of *K. senegalensis* (Lompo et al., 1998; Lompo et al., 2007). Limonoids were identified and isolated in the leaves and stem bark of *K. senegalensis* (Zhang et al., 2009; Yuan et al., 2012).

The aim of the present study was to evaluate the antioxidant activity of aqueous ethanol extract and its fractions (n-hexan, ethyl acetate, n-butanol and aqueous) of *K. senegalensis* stem barks, and this study was to determine total phenolic, tannins, flavonoids and flavonol contents in the extract and its fractions.

MATERIALS AND METHODS

Chemicals and reagents

ABTS (2, 2'-azino-bis(acide 3-ethylbenzothiazoline-6-sulfonique), DPPH (2,2-diphényl-1-picrylhydrazyl), trolox, quercetin, sodium

acetate, Folin-Ciocalteu reagent (FCR 2N), polyvinylpolypyrrolidone, aluminum chloride and potassium persulfate were purchased from Sigma (St Louis, USA). Trichloroacetic acid and 2-thiobarbituric acid were from Fluka chemica. Potassium hexacyanoferrate [$K_3Fe(CN)_6$] were purchased from Prolabo (Paris, France).

Plant

The present study was undertaken on the stem bark of *K. senegalensis*, which were collected in May, 2011 at Samogohiri, in Kenedougou district (West region of Burkina Faso). The plant was identified by Dr Ouédraogo Amadé, a Botanist at the Department of Forest of INERA/CNRST-Burkina Faso. A voucher specimen was deposited at the National Herbarium of CNRST with number ID16879 and GPS data (10°39'14.25 N; 4°39'52.96 W).

Preparation of plant extract and fractions

Five hundred grams (500 g) of powder of stem bark of *K. senegalensis* were macerated with 2.5 L of 80% (v/v) of aqueous ethanol (96%) for 24 h at 25°C. The resulting mixture was filtered using paper Whatman (N°1) and then was evaporated to dryness under reduced pressure in a rotary evaporator (Büchi 461, Switzerland) at 45°C to yield crude aqueous ethanol extract (69 g). Aqueous ethanol extract (AEE) (34.5 g) suspended in water (500 ml) was partitioned with n-hexan (3 × 200 ml), ethyl acetate (3 × 200 ml) and n-butanol (3 × 200 ml) to obtain a n-hexan fraction (0.61 g), an ethyl acetate fraction (1.88 g), n-butanol fraction (1.51 g) and aqueous fraction (13.1 g).

Antioxidant activity determinations

DPPH• assay

DPPH• radical scavenging activity was done according to Kim et al. (2003). 10 µl of extract or fractions or standard was added to 200 µl of DPPH methanolic solution (0.04 mg ml⁻¹) in a 96-well microtitre plate and vortexed. After 30 min incubation in the dark at room temperature, the absorbance was measured at 490 nm using spectrophotometer BioRAD (model 680, Japan). Each determination was carried out in triplicate. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and expressed as antiradical power (ARP = 1/EC₅₀).

DPPH' assay on thin layer chromatography (DPPH-TLC)

Aqueous ethanol extract and fractions of *K. senegalensis* stem bark were applied using Silica gel 60 F₂₅₄ plates (Merck). The mobile phase was butanol-glacial acetic acid-water (60-20-20; V/V/V). Sample (10 mg ml⁻¹, 10 µl) were directly deposited as spot onto the TLC plates. After deposition of sample, the plates were dried and placed in migration chamber previously containing eluent. On the plate, the distance of the eluent path was 80 mm from the point of deposit spot. After migration, the plates were removed and dried at room temperature for 30 min. Detection of antioxidant compounds was achieved by spraying plates with a DPPH in methanol. The presence of antioxidant compounds was detected by yellow spots.

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ABTS^{•+} assay

ABTS^{•+} radical scavenging assay was used to determine the capacity antioxidant of extract or fractions according to Re et al. (1999). ABTS^{•+} diammonium salt solution (75 mM) and potassium persulfate (1.225 mM) were mixed overnight. The mixture was diluted with ethanol (96%) before assay. 200 μ l of radical ABTS^{•+} solution were added to 20 μ l of extract or fractions in 96-well microplate. After 30 min incubation in the dark at 25°C, the absorbances were measured at 734 nm using spectrophotometer BioRad (model 680, Japan). Data obtained were the means of three determinations. The capacity antioxidant using ABTS method was expressed as trolox equivalent antioxidant capacity (TEAC).

Ferric reducing antioxidant power (FRAP) assay

FRAP was determined in extract or fractions by method described by Apati et al. (2003). 0.5 ml of extract or fractions (1 mg mL⁻¹) was mixed with 1.25 ml of phosphate buffer and 1.25 ml of aqueous solution of potassium hexacyanoferrate (1%). After 30 min of incubation at 50°C, 1.25 ml of trichloroacetic acid (10%) was added to mixture. After centrifugation at 3000 g during 10 min, the upper layer solution (0.625 ml) was mixed with distilled water (0.625 ml) and FeCl₃ solution (0.125 ml, 0.1%). Absorbances were recorded at 700 nm using spectrophotometer Agilent (Agilent 8453, USA) equipped with UV-visible ChemStation software. Trolox was used to produce calibration curve ($R^2 = 0.99$). FRAP activity of samples was carried out in triplicate and expressed in mmol trolox equivalent/gram of extract.

Lipid peroxidation inhibition (LPO)

The inhibition activity of extract or fractions on lipid peroxidation was determined according to method described by Ohkawa et al. (1979) using thiobarbituric acid. Briefly, 40 μ l of extract or fractions (10 mg mL⁻¹) was mixed with 200 μ l of rat liver homogenate (1%), 10 μ l of FeCl₂ (0.5 mM) and 10 μ l of H₂O₂ (0.5 mM). After 60 min incubation at 37°C, 200 μ l of trichloroacetic acid (15%) and 200 μ l of 2-thiobarbituric acid (0.67%) were added to mixture. Then, the final mixture was heated up in boiled water during 15 min. The absorbances were measured at 532 nm using spectrophotometer Agilent (Agilent 8453) equipped with UV-visible ChemStation software.

Phytochemical investigations

Determination of total phenolic content

The method of Singleton et al. (1999) using Folin-Ciocalteu reagent (FCR 2N) was used to determine the total phenolic contents. 1 ml of aqueous ethanol extract or fractions (0.5 mg mL⁻¹) was mixed with 1 ml of FCR 2N and 3 ml of sodium carbonate (20%, w/v). The mixture obtained was incubated for 40 min at room temperature. After incubation, the absorbances were recorded at 760 nm with spectrometer Agilent (Agilent 8453) equipped with UV-visible ChemStation software. Tannic acid was used as a standard; results were expressed as milligram of tannic acid equivalent (mg TAE)/g of extract. Data presented are average of three measurements.

Determination of tannins content

Tannins content was determined using Folin-Ciocalteu reagent described by Singleton et al. (1999) as aforementioned, after precipitating the phenolic with polyvinylpyrrolidone (PVPP). 100

mg of PVPP was mixed with 1 ml of extract or fractions (10 mg mL⁻¹) in test tube. After 15 min incubation at 4°C, tubes were vortexed and centrifuged for 10 min at 3000 g. Two (2) ml of supernatant of each tube were sampled and been used to determine phenolic content as described above (method of phenolic content determination). Tannins contents were calculated subtracting from total phenolic contents and these are expressed as tannic acid equivalent (Tibiri et al., 2007). The amount of tannins was determined as the difference between total phenolics (containing tannins) and the total phenolics (in absence of tannins)

Determination of total flavonoids content

The total flavonoids content was determined according to the method described by Abdel-Hamed (2009). 100 μ l of extract or fractions (10 mg mL⁻¹) were mixed with 100 μ l of aluminum chloride (2%). After 40 min, the absorbance was recorded at 415 nm against a blank using spectrometer Agilent (Agilent 8453) equipped with UV-visible ChemStation software and compared to quercetin calibration curve ($R^2 = 0.99$). Results obtained were the means of three determinations. Total flavonoid content was expressed as milligrams of quercetin equivalent (mg QE) per g of extract or fraction.

Determination of flavonol content

Flavonol content was determined according to the method described by Abdel-Hameed (2009). 1 ml of extract or fractions (10 mg mL⁻¹) was mixed with 1 ml of aluminium trichloride (20 mg mL⁻¹) and 3 ml of sodium acetate (50 mg mL⁻¹). After 2 h 30 min of incubation, the absorbance was measured at 440 nm. Quercetin was used as standard compound. All results were obtained in triplicate. Data of flavonol content was expressed as g of quercetin equivalent (g QE)/100 g of extract or fraction.

Statistical analysis

All tests of antioxidant activity and determination of compound contents were conducted in triplicates. Data obtained were expressed as mean $\pm$ standard deviation (SD) of three replicates. Statistical comparison of data was performed by one-way analysis of variance (ANOVA) using Graph Prism version 5.0 software. P value < 0.05 were considered.

RESULTS AND DISCUSSION

Antioxidant activity of aqueous ethanolic extract (AEE) of stem bark of *K. senegalensis* and its fractions was measured using DPPH[•], ABTS^{•+}, FRAP and Lipidic peroxidation (LPO) methods. Liquid partition was allowed to obtain four fractions from aqueous ethanolic extract, using solvents such as n-hexan, ethyl acetate, n-butanol and water. Polar and non-polar fractions of *K. senegalensis* could be worthwhile in order to find a correlation between the antioxidant and the phenolic contents. The antioxidant activity using five different methods (DPPH, DPPH-TLC, ABTS, LPO, FRAP) of aqueous ethanolic extract and fractions are summarized in Table 1.

DPPH[•] radical scavenging activity was evaluated in terms of percentage inhibition of a pre-formed free radical

Table 1. Antioxidant activity of aqueous ethanol extract and fractions of *K. senegalensis* stem bark.

Samples	ABTS (TEAC)	FRAP (mmol TE/g)	Lipid peroxidation inhibition (%)	DPPH IC ₅₀ (μg ml ⁻¹) (ARP)
Aqueous ethanol extract	3 ± 0.05*	13.40 ± 0.05*	57.08 ± 1.06*	2.3 ± 0.2 (0.43)*
n-Hexan fraction	8478 ± 0.3*	---	49.65 ± 1.61	170.3 ± 0.2 (0.006)*
Ethylacetate fraction	166 ± 0.2*	13.04 ± 0.25*	70.30 ± 0.40*	7.6 ± 0.15 (0.13)*
Butanol fraction	4 ± 0.2*	13.60 ± 0.09*	58.70 ± 0.80*	1.76 ± 0.2 (0.56)*
Aqueous fraction	3.01 ± 0.2*	13.55 ± 0.10	61.72 ± 0.00*	2.05 ± 0.2 (0.49)*
Quercetin	---	---	47.92 ± 0.001*	1.06 ± 0.13 (0.94)
Gallic acid	---	---	43.14 ± 0.43*	---

Values are mean ± S.E.M. for triplicate; *: $P < 0.05$ significant from control (one-way ANOVA analysis followed by Dunnett's test); ARP (antiradical power) = $1/IC_{50}$; TEAC: trolox equivalent antioxidant capacity. TE: trolox equivalent

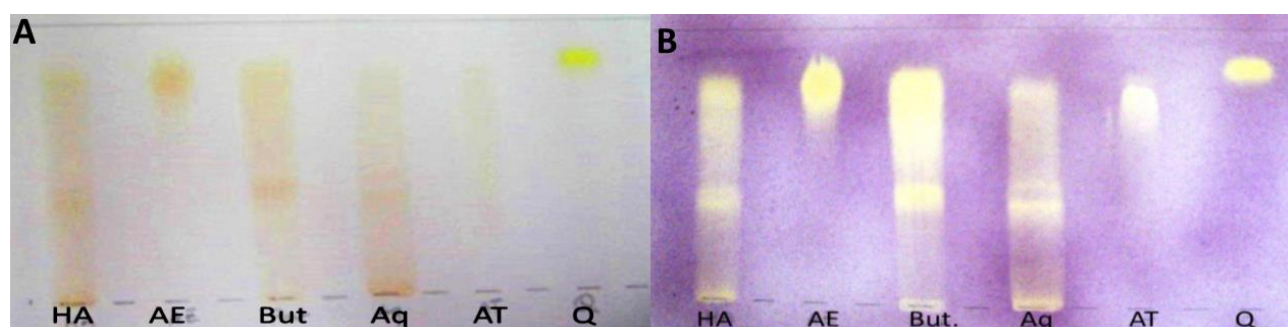


Figure 1. TLC plates photography of extract, fractions and standards before (A) and after (B) sprayed with DPPH solution. HA: aqueous ethanol extract, AE: ethyl acetate fraction, But: n-butanol fraction, Aq: aqueous fraction, AT: tannic acid and Q: quercetin.

by antioxidants compounds in extract and fractions. Aqueous ethanolic extract and fractions exhibited free radical scavenging effect in concentration dependent manner. The IC₅₀ values ranged from 1.76 ± 0.19 to $170.30 \pm 0.20 \mu\text{g ml}^{-1}$. Butanol fraction had the highest value with $1.76 \pm 0.19 \mu\text{g ml}^{-1}$ (ARP = 0.56) against n-hexan fraction with $170.30 \pm 0.20 \mu\text{g ml}^{-1}$ (Table 1). Ibrahim et al. (2014) reported that ethanol extract of *K. senegalensis* stem bark contained polyphenols and possessed antioxidant activity (IC₅₀ = $1.99 \pm 0.87 \mu\text{g ml}^{-1}$) using DPPH assay. This value is not significantly different than obtained value. Comparing these results with others species of *Khaya* genus, *K. senegalensis* possess high antioxidant activity than methylene chloride/methanol (1:1 v/v) extract of *Khaya grandifoliola* C. DC (Meliaceae) stem barks with IC₅₀ = $4.54 \pm 0.28 \mu\text{g ml}^{-1}$ using DPPH assay (Njayou et al., 2015).

TLC-DPPH• test was used to evaluate the qualitative antioxidant of extract and fractions from *K. senegalensis* stem bark. TLC-DPPH• assay indicated the presence of the antioxidant compounds in the extract and fractions. In Figure 1, the yellow spots observed were antioxidant compounds. The yellow spots were: one majory spot (Rf = 0.78) with ethyl acetate fraction, two spots with aqueous ethanol extract: (Rf = 0.36, 0.77), butanol

fraction (Rf = 0.78, 0.39), and aqueous fractions (Rf = 0.36, 0.75), quercetin (Rf = 0.85), tannic acid (Rf = 0.72). TLC-DPPH assay revealed the antioxidant compounds while DPPH assay using spectrophotometer gave the antioxidant activity of whole extract. TLC-DPPH• tests, reported in literature, focus on phenolic compounds such as flavonoids, phenolic acid, tannins (Ciesla et al., 2012).

Like DPPH assay, ABTS•⁺ assay is widely used to determine antioxidant activity of substances. ABTS•⁺ radical scavenging activity of extract and fractions was expressed in TEAC values which ranged from 8478 ± 0.3 to 3 ± 0.05 . N-hexan fraction showed the highest capacity to scavenge ABTS•⁺ (Table 1). Variation of ABTS•⁺ radical scavenging activity may be associated to the different constituents in each extract; aqueous ethanolic extract activity could be due to the tannins contents (Khan et al., 2012) and n-hexan fraction activity could be due to the presence of lipophilic compounds (Osman et al., 2009).

FRAP values varied from 13.04 ± 0.25 to 13.60 ± 0.09 mmol Trolox Equivalent per gram (mmol TE/g) of extract or fraction (Table 1). The highest FRAP value was obtained with ethyl acetate fraction (13.04 ± 0.25 mmol TE g⁻¹); however there is not significant difference between FRAP values of fractions and extract. At a

Table 2. Total phenolics, tannins, flavonoids and flavonols contents of *K. senegalensis* stem bark.

Samples	total phenolic (g TAE/100 g dw)	Tannins (g TAE/100 g dw)	Total flavonoid (g QE/100 g dw)	Flavonol (g QE/100 g dw)
Aqueous ethanol extract	9.36±0.53*	5.78±0.44*	0.11±0.02*	0.36±0.04*
n-Hexan fraction	0.01±0.001*	0.01±0.001*	0.001±0.001*	-
Ethylacetate fraction	0.33±0.04*	0.04±0.001**	0.01±0.001*	0.001±0.001
Butanol fraction	0.49±0.03*	0.25±0.02*	0.01±0.001*	0.01±0.001*
Aqueous Fraction	3.68±0.11*	2.65±0.18*	0.04±0.01	0.10±0.01*

Values are mean ± S.E.M. for triplicate; *: $P < 0.05$ significant from control (one way ANOVA analysis followed by Dunnett's test); TAE : tannic acid equivalent; QE : quercetin equivalent; dw : dried weight.

concentration of $100 \mu\text{g ml}^{-1}$, the crude extract and its fractions showed by the lipid peroxidation test inhibition values ranged from 49.65 ± 1.61 to $70.30 \pm 0.40\%$. Ethyl acetate fraction presented the highest activity ($70.30 \pm 0.40\%$) and the lower activity was given by n-hexan fraction (49.65 ± 1.61). In addition, inhibitor effect of ethyl acetate fraction against lipid peroxidation was more than the standard compounds gallic acid ($43.14 \pm 0.43\%$) and quercetin ($47.92 \pm 0.01\%$).

In pathological conditions, the excessive production of free radical provokes the induction of lipid peroxidation leading to cell damaging. Lipid peroxidation inhibition allows the prevention of cell lysis inhibiting free radical. The test of lipid peroxidation inhibition method allowed to obtain percentage inhibition varied from 49.65 ± 1.61 to $70.30 \pm 0.40\%$ at same concentration ($100 \mu\text{g ml}^{-1}$). Ethyl acetate fraction presented the best activity ($70.30 \pm 0.40\%$) and lower activity was n-hexan fraction ($49.65 \pm 1.61\%$). In addition, inhibitory effect of ethyl acetate fraction against lipid peroxidation was more than standard compounds such as gallic acid ($43.14 \pm 0.43\%$) and quercetin ($47.92 \pm 0.001\%$).

The literature for antioxidant activity of *K. senegalensis* using ABTS, FRAP and LPO methods has not been found, however the antioxidant activity of *K. senegalensis* was measured using deoxyguanosine, hydroxyl radical (HRS) and Nitric oxide (NO) radical scavenging models (Atawodi et al., 2009 ; Ibrahim et al., 2014).

One method is not sufficient to evaluate that antioxidant capacity but it takes more than one method to take into account different modes of action of antioxidants (Dudonné et al., 2009). This study showed that the most active fraction depends on the method used; n-hexan fraction was more active than other fractions in ABTS $^{\bullet+}$ assay; however, in DPPH $^{\bullet}$ assay, n-butanol fraction was more active. This could be due to different mechanisms involved in the steps of oxidation process and antioxidant composition such as secondary metabolites (Conforti et al., 2009). The study found that n-hexan fraction containing lipophilic compounds was more active with ABTS $^{\bullet+}$ assay. According to Prior et al. (2005), hydrophilic and lipophilic compounds act against ABTS $^{\bullet+}$ radical. In addition, the antioxidant activity depends on

the amount of compounds that react with the free radical formed in each method used.

The total phenolics, tannins, total flavonoids and flavanol contents of extract and fractions are shown in Table 2. Aqueous ethanolic extract of *K. senegalensis* contains total phenolic, tannins, total flavonoids and flavanol. Among fractions, aqueous fraction had the highest of total phenolics and tannins contents with, respectively 3.68 ± 0.11 and 2.65 ± 0.18 g TAE/100 g of dry weight (dw) of plant material, followed by n-butanol fraction. Aqueous fraction also showed the highest of total flavonoids (0.04 ± 0.01 g QE/100 g dw) and flavanol (0.10 ± 0.01 g QE/100 g dw) contents. Phenolic contents have already been reported in stem barks extracts of *K. grandifoliola* (Njayou et al., 2015) and *K. senegalensis* (Ibrahim et al., 2014).

The antioxidant effect of substances is important to prevent, to delay or to treat oxidative stress involved in pathogenesis of many chronic pathologies including cancer, cardiovascular diseases, arthritis, diabetes. Several study has reported the antioxidant activity of phenolic compounds such as polyphenolic, tannins and flavonoids. Antioxidant activity of these compounds is due to their oxidation-reductive property, which play an important role in the adsorption and neutralization of free radical (Manish et al., 2011; Ouédraogo et al., 2012).

Previous studies had reported a strong correlation between antioxidant activity and phenolic compounds present in the extracts from medicinal plants (Wang et al., 2016; Dudonné et al., 2009). The analysis of data significantly revealed a correlation observed between DPPH $^{\bullet}$ method and total phenolic ($R^2 = 0.98$, $p < 0.05$) (Figure 2) and flavonols ($R^2 = 0.98$, $p < 0.05$). The antioxidant activity of *K. senegalensis* stem bark is due to the synergic action of different compounds which act by direct free radical scavenging, chelation of transition metal and direct inhibition of lipid peroxidation.

Conclusion

The present study showed an interesting antioxidant potential of aqueous ethanol extract and different

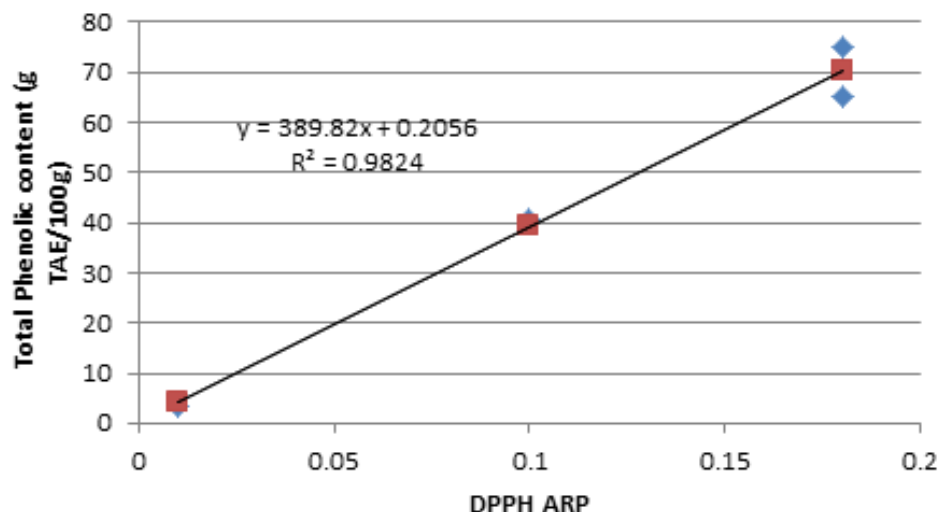


Figure 2. Correlation between total phenolic content and antioxidant activity using DPPH assay.

fractions obtained of stem bark of *K. senegalensis* A. Juss (Meliaceae). Stem bark of *K. senegalensis* contains total phenolic, tannins and flavonoids. These results provide scientific evidence that validates the use of *K. senegalensis* in traditional medicine.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This work was supported in 2010 by the Program of C.U.D (Belgium) for Post-doctoral research. This research was carried on both in Belgium and in Burkina Faso in the Department of Traditional Medicine of IRSS/CNRST-Burkina Faso. The author wish to thank Dr Amadé OUEDRAOGO to plant identification.

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