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Alioune Dior Fall
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. 5005 Dakar-Fann. Dakar, Senegal

Serigne Ibra Mbacké Dieng
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. 5005 Dakar-Fann. Dakar, Senegal

Kady Diatta-Badji
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. 5005 Dakar-Fann. Dakar, Senegal

William Diatta
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. 5005 Dakar-Fann. Dakar, Senegal

Emmanuel Bassène
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. 5005 Dakar-Fann. Dakar, Senegal

Correspondence
Alioune Dior Fall
Laboratory of Pharmacognosy and Botany. Cheikh Anta Diop University. BP 5005 Dakar-Fann. Dakar, Senegal

Phytochemical screening, phenol content and antioxidant studies of ethanol leaf extract of *Celtis toka* (Forssk.) Hepper & J.R.I. Wood

Alioune Dior Fall, Serigne Ibra Mbacké Dieng, Kady Diatta-Badji, William Diatta and Emmanuel Bassene

Abstract
The present study aimed to identify the phytochemical groups of leaf extract of *Celtis toka* Forssk.) Hepper & J.R.I. Wood, to evaluate the phenol content of extracts and their antioxidant activity. Coumarins, flavonoids, mucilage, triterpenoids and steroids were the main phytochemical constituents of the leaf extract. The ethanol extract and its fractions were found to be active on the 4 antioxidant methods. On ABTS assay the ethanol extract (IC_{50} : 48.6±6.8 µg/ml) was more active than its three fractions while on nitric oxide test the said extract had similar effect than the more active fraction (aqueous fraction). The ethyl acetate and the chloroform fractions were found to be more active on DPPH and FRAP tests respectively.

Keywords: *Celtis toka*, leaf, phytochemical, phenolic, antioxidant

1. Introduction
Medicinal plants are known for their activity against oxidative stress induced by free radicals overproduction.
Overproduced free radicals react by oxidizing bio-molecules such as nucleic acids, proteins, lipids and DNA and lead to degenerative diseases like neurological disorders, arthritis, atherosclerosis, cancer [1]. Human body has an antioxidant system including enzymes such as superoxide dismutase and catalase which can protect against the damaging effects of these molecules. Polyphenols from medicinal plants, known for their antioxidant capacity, can also limit the onset of these diseases. What makes us to evaluate the phenol content and the antioxidant activity of leaf extract of *Celtis toka* Forssk.) Hepper & J.R.I. Wood and its fractions.
Celtis toka (synonym*: Celtis integrifolia*) [2] is a plant of the *Ulmaceae* family measuring 15-20 m high. The leaves are alternate, more or less tough on both sides, ovate with acuminate or attenuated peak. The leaves are used in Senegalese traditional medicine to treat asthenia, measles, abscesses, fungal infections and edema. They are also used as vegetables [2]. However, there are not available studies in the chemistry or biological activity of this plant.
This study aimed to investigate the phytochemical composition, the phenol content and the antioxidant activity of ethanol leaf extract of *C. toka* and its chloroform, ethyl acetate and aqueous fractions.

2. Material and methods
2.1 Plant collection
Leaves of *Celtis toka* were collected at Thies (Senegal). The plant was identified and authenticated by Dr W. Diatta (Herbarium of the Botanical Garden of the Faculty of Medicine, Pharmacy and Odontology of Dakar). Voucher specimen were kept at the said herbarium. Plant leaves were washed with distilled water and air dried at room temperature. Dried leaves were ground to a fine powder.

2.2 Extraction and fractionation
Powdered leaves of *Celtis toka* (60 g) were decocted twice for 30 minutes using 1 l of ethanol and filtered through filter paper. Ethanol was removed under reduced pressure using a rotary evaporator leading to the ethanol extract (EE). For liquid/liquid fractionation, 2 g of dried ethanol extract was dissolved in a mixture (distilled water/ chloroform; 1:1). After decantation in a separatory funnel, the aqueous solution obtained was extracted twice with chloroform. The chloroform solutions were combined to give the chloroform fraction (CHF).

The aqueous solution was again subjected to liquid-liquid extraction with ethyl acetate under the same conditions as above. The ethyl acetate and aqueous solutions obtained were evaporated separately and lead to the corresponding fractions (EAF and AF).

2.3 Phytochemical screening
Standard phytochemical analyses were carried out to test for the presence of the phytoconstituents in the prepared extract. Chemical tests were carried out on ethanol and aqueous extracts of the powdered specimens using standard procedures for the detection of alkaloïds (Bouchardat, Valser-Mayer and Dragendorff's reagents tests), cardiac glycosides (Baljet, Kedde and Raymond-Marthoud reagents tests), saponins (foaming index), carotenoids (antimony chloride/chloroform test), steroids and triterpenoids (Liebermann-Buchard test), anthracenic glycosides (Borntraeger test), tannins (Stiasny test followed by ferric chloride test), flavonoids (Shibata's test), reducing sugars (Fehling's test), coumarins (ammonia solution / UV), mucilage (Ruthenium red test) in order to identify the presence of phytochemical constituents [3-4].

2.4 Phenol content (PC)
Total phenol contents of samples were investigated using the reported method slightly modified [5]. A mass of dried sample (2.5 mg) was dissolved in 100 ml of distilled water. An aliquot (10 ml) of this solution was mixed with Folin Denis reagent (2 ml), 15% sodium carbonate (2 ml) and centrifuged for 4 minutes at 4000 rpm. Absorbance of the blue solution was measured at 760 nm. A stock solution of tannic acid was prepared by dissolving 5 mg in 100 ml of distilled water. Then two-fold serial dilutions of tannic acid solution were made before adding Folin Denis reagent and 15% sodium carbonate. Absorbance was measured at 760 nm and plotted against concentrations. All experiments were done in triplicate and results were expressed as milligrams of tannic acid equivalents (TAE) per gram of dried sample (extract or fractions).

2.5 Antioxidant activity
2.5.1 ABTS assay
ABTS (2, 2-azinobis-3- ethylbenzothiazoline-6-sulfonic acid) assay was assessed using the described protocol [6]. A solution of potassium persulfate (2.45 mM) was mixed at equal volumes with ABTS (7 mM) solution. The mixture was incubated for 12 h at room temperature in darkness and lead to a stock solution. For assays a freshly prepared solution was used by adding to the stock solution 50% methanol in order to have an initial absorbance of about 0.700±0.02 at 745 nm. ABTS free radical scavenging ability of sample was evaluated by adding 50 µl of each sample at appropriate concentration to 1.5 ml of ABTS working solution. The absorbance decreasing was measured 10 min after. All experiments were done in triplicate. The ABTS free radical scavenging effect was expressed as IC_{50} (concentration of sample required to scavenge 50% of free radicals).

2.5.2 DPPH assay
The determination of the DPPH free radical scavenging activity of samples was done using the described method [7]. An ethanol solution of DPPH was prepared by dissolving 4 mg in 100 ml of ethanol. An aliquot of each sample (0.8 ml) at appropriate concentration was added to 3 ml of ethanol solution of DPPH. The ethanol leaf extract of *Celtis toka*, its fractions (chloroform, ethyl acetate and water) and ascorbic acid were tested at different concentrations. The absorbance of each sample was measured at 517 nm after 30 min. Each experiment was done in triplicate and the absorbance of the initial ethanol DPPH solution did not change after 30 min. The antioxidant activity related to the DPPH free radical scavenging effect was expressed as IC_{50} (concentration of sample required to scavenge 50% of free radicals).

2.5.2 Nitric oxide (NO) assay
Reduction of free radical NO was determined using the colorimetric assay described [8]. A 13.8 mM nitroprusside solution (1 ml) was added to distilled water (250 µl) and an aliquot of each sample (250 µl) at appropriate concentration. The mixture was incubated at room temperature for 150 min. Then, 1 ml of sulfanilic acid solution (0.5% in 20% glacial acetic acid) was added to 500 µl of the solution cited above. The resulting mixture was incubated again for 5 minutes.
Finally, 1 ml of aqueous solution of N-(1-Naphtyl) ethylenediamine dihydrochloride (0.038 mM) was added to the above solution; after incubation for 30 minutes the absorbance was measured at 540 nm. Ascorbic acid was used as positive control and each concentration of sample was tested in triplicate. The antioxidant activity related to the nitric oxide free radical scavenging effect was expressed as IC_{50} (µg/ ml) representing the concentration of the sample that caused 50% inhibition.

2.5.3 Ferric reducing antioxidant power assay (FRAP)
The ferric reducing power was determined according to the described method [9]. An aliquot of 0.20 ml of each sample at appropriate concentration was mixed with 0.5 ml of phosphate buffered saline (0.2 M; pH 6.6) and 0.5 ml of 1% potassium ferricyanide (K 3Fe(CN) 6). The mixture was incubated at 50°C for 30 min and 0.5 ml of 10% trichloroacetic acid was added. After centrifugation for 10 minutes at 3000 rpm, the supernatant (0.5 ml) was mixed with distilled water (0.5 ml) and 0.1% ferric chloride (0.1 ml). Absorbance was measured at 700 nm; ascorbic acid was used as positive control. Absorbance increasing relatively to that of concentration represented the reducing capacity of tested sample.

2.5.4 Statistical analyses
Data were expressed as mean± SD. Analyses of variance (ANOVA) were done for the comparison of results using Fischer's test. Statistical significance was set at $p < 0.05$.

3. Results
3.1 Extraction
After extraction of 60 g of powdered leaf material with ethanol, a mass of 6.6 g of dried extract was obtained representing a yield of 11 % w/w. Chloroform, ethyl acetate and water fractions represented respectively 17.5- 6.5- 55.5% w/w of the dried ethanol leaf extract.

3.2 Phytochemical screening
Phytochemical screening revealed that steroids, triterpenoids, flavonoids, coumarins and mucilage were the main constituents of the ethanol leaf extract of *C. toka*. Steroids, triterpenoids and coumarins were found in chloroform and ethyl acetate fractions while mucilage and flavonoids were detected in polar fractions (ethyl acetate and aqueous fractions). Negative reactions were obtained for the presence of carotenoids, alkaloids, tannins, saponins (foaming index less than 100), reducing sugars, anthracenic derivatives and cardiac glycosides (Table 1).

Table 1: Phytochemical groups identified in leaf extract of *Celtis toka* and its fractions.

Phytochemical groups	EE	CHF	EAF	AF
Alkaloids	-	-	-	-
Cardiac glycosides	-	-	-	-
Saponins	-	-	-	-
Carotenoids	-	-	-	-
Steroids and triterpenoids	+	+	+	-
Anthracenic glycosides	-	-	-	-
Tannins	-	-	-	-
Flavonoids	+	-	+	+
Coumarins	+	+	+	-
Reducing sugars	-	-	-	-
Mucilage	+	-	+	+

+ : presence ; - : absence

3.3 Phenol content

The ethyl acetate fraction (PC: 58.8±4.76 mg ETA/g) had higher phenol content than the ethanol leaf extract of *C. toka* (PC: 43.7±5.78 mg ETA/g; p<0.005), the aqueous and ethyl acetate fractions (PC: and 38. 2±4.23 mg ETA/g respectively). The chloroform fraction (7±1.01 mg ETA/g) was found to have the smallest phenol content, as shown in figure 1 (p<0.001).

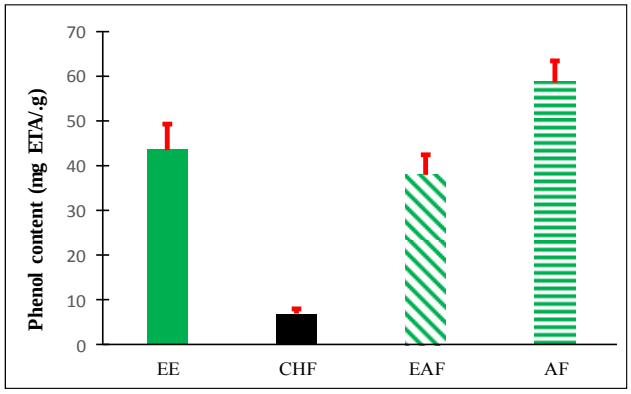
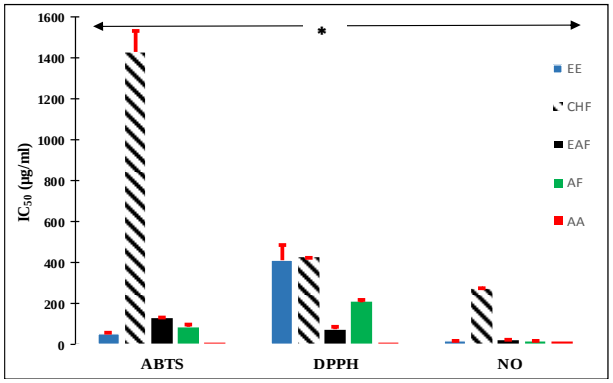


Fig 1: Phenol content of ethanol leaf extract and fractions of *C. toka*.

3.4 Antioxidant activity

3.4.1 ABTS assay

The chloroform, ethyl acetate and aqueous fractions (with respective IC_{50} values: 1428.8±106.4-125±3.07-83±11.73 µg / ml) were found to be less active than the ethanol leaf extract (IC_{50}: 48.6±6.8 µg/ml) (p<0.05). Ascorbic acid had the lowest IC_{50} (4.04±0.31 µg/ml), (Fig. 2).



*: significant difference (p<0.05 versus negative control).

Fig 2: IC_{50} values of different tested samples on ABTS, DPPH and NO reducing assays.

3.4.2 DPPH assay

The ethyl acetate fraction (IC_{50}: 72±11.98 µg/ml) and the aqueous fraction ($IC_{50:}$ 205.8±8.5 µg/ml) exhibited better ability to inhibit the free radical DPPH than the ethanol extract (410.4 ±73.74 µg/ml) (p<0.05). The chloroform fraction (IC_{50} : 423.1±13.61 µg/ml) and ethanol extract had shown similar activity while ascorbic acid had exhibited the better ability to inhibit the free radical DPPH (see Fig. 2).

3.4.3 NO assay

The ethanol extract (IC_{50}: 13.2±2.63 µg/ml) and the aqueous fraction (IC_{50}: 14.33±0.53 µg/ml) had exibited similar capacity to scavenge the nitric oxide than ascorbic acid (IC_{50}: 12.4±0.25 µg/ml). The ethyl acetate fraction (IC_{50}: 16.86±1.86 µg/ml) was more active than the chloroform fraction (IC_{50}: 270.2±4.75 µg/ml) (p<0.001), as shown in Fig 2.

3.4.4 FRAP assay

As shown in Figure 3, for all samples, increasing absorbance were noticed relatively to the concentration variations (p<0.05). Chloroform and ethyl acetate fractions exhibited higher ferric reducing power than the ethanol extract of *C. toka*. At all tested concentrations, the extract had shown better ability to reduce ferric ion than the aqueous fraction. Ascorbic acid tested at different concentrations than the other samples had an important ferric reducing capacity (Fig. 3).

The absorbance values of the chloroform and ethyl acetate fractions varied respectively from 0.369±0.08 – 0.455±0.04 at 63 µg/ml to 0.620±0.06 – 0.591±0.05 at 100 µg/ml while those of ascorbic acid increased from 0.17±0.016 at 6.25 µg/ml to 0.67±0.009 at 100 µg/ml.

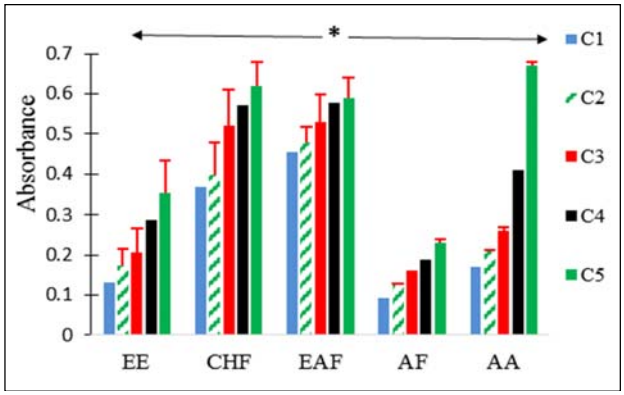


Fig 3: Ferric reducing capacity of different samples.

EE (Ethanol extract), CHF (Chloroform fraction), EA (Ethyl acetate fraction), AF (Aqueous fraction): C1 =63 µg/ml; C2 =189 µg/ml; C3 =315 µg/ml; C4 =441 µg/ml; C5 =567 µg/.

AA (Ascorbic acid): C1 =63 µg/ml; C2 =189 µg/ml; C3 =315 µg/ml; C4 =441 µg/ml; C5 =567 µg/ml.

*: significant difference (p<0.05)

4. Discussion

The polar fractions (ethyl acetate and aqueous fractions) represented 62% of the mass of the ethanol extract. The polar constituents were quantitatively more present than the apolar compounds.

Phytochemical groups identified in the extract and fractions such as triterpenoids [10-11], flavonoids [12], coumarins [13] and mucilage [14] had been described for their antioxidant ability.

These phytochemical groups had been found in *Celtis* genus plants such as *Celtis iguanaea* [15] and *C. australis* [16].

The apolar fraction (chloroform fraction) had shown better ability to reduce the ferric ion on FRAP assay. What makes us to suggest that apolar constituents present on the chloroform fraction had better ferric reducing capacity than the polar components.

Meanwhile on DPPH, ABTS and nitric oxide scavenging assays, the chloroform fraction had exhibited lower activity than the leaf extract, the ethyl acetate and aqueous fractions of *C. toka*. The phenol contents of the leaf extract and the polar fractions (ethyl acetate and aqueous fractions) were higher than that of the apolar chloroform fraction. The ethanol leaf extract of *C. toka* (PC: 43.7±5.78 mg ETA/g) had higher polyphenol content than leaf and stems methanol extracts of *Celtis africana* (PC: 14±0.11 and 15.39±0.28 mg equivalent of tannic acid /g of dried material respectively). The evaluation of flavonoids content of leaf extract of *Celtis africana* (0.70 ± 0.02 mg equivalent of quercetin/g) had shown that these constituents may represent an important part of the total polyphenol content [17].

Polyphenol compounds are known for their antioxidant effect and could then be partly responsible for the antioxidant activity of the extract and polar fractions. The antioxidant activity of phenolic compounds is related to their redox properties, which permit them to adsorb and to neutralize free radicals, to quench singlet and triplet oxygen, or to decompose peroxides [18]. On DPPH test, antioxidants gave hydrogen radical to stabilize it and form the DPPH-H [19]. On the nitric oxide reducing test, antioxidant compounds trapped nitric oxide in competition with oxygen and reduced production of nitric oxide which has a pro-oxidant effect. NO radical is unstable in aerobic conditions and produces genotoxic intermediates such as nitrites, nitrates and peroxynitrites reacting with oxygen and superoxide [20].

The leaf extract of *C. toka* and its fractions had better ability to scavenge the nitric oxide than ABTS and DPPH free radicals. Moreover the ethanol leaf extract and the aqueous fraction were found to exhibit better capacity to inhibit the ABTS than the DPPH free radicals.

The solubility of the samples in the different testing solutions may affect their ability to inhibit free radicals. The antioxidative study of phenolic constituents from *Salvia officinalis* had revealed that compounds which scavenged ABTS radical did not inhibit the radical DPPH [21].

Anti-oxidants agents are known for the major role they play in preventing various diseases such as Alzheimer's disease, cancer, diabetes, cardiovascular diseases.

5. Conclusion

In this study, ethanol leaf extract of *Celtis toka* which contained flavonoids, coumarins, triterpenoids and steroids had exhibited antioxidant activity on ABTS, DPPH, NO and FRAP assays. Isolation of compounds from active fractions may be the focus of further studies.

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