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Kengni Yande H
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Toghueo KRM
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Hzounda Fokou JB
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Adamou Souleymanou
Laboratory of Biotechnology, Biotechnology center, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon. Nkolbisson, Cameroon.

Ngouana V
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Fekam Boyom F
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Correspondence
Kengni Yande H
Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon.

Phenol content and Antioxydant activity of crude extracts and fractions from *Annona senegalensis*

Kengni Yande H, Toghueo KRM, Hzounda Fokou JB, Adamou Souleymanou, Ngouana V and Fekam Boyom F

Abstract
This study was designed to analyse the antioxidant potential and phenol content of ethanol extract and fractions from *Annona senegalensis*, a medicinal plant used worldwide against several oxidative stress mediated disorders. The antioxidant activities were determined by three methods namely DPPH radical scavenging assay, ferric reducing antioxidant power (FRAP) and nitric oxide inhibition (NO). The antioxidant activities ranged from IC_{50} 1.5 to 167.00µg/ml for DPPH, from 0.53->200µg/ml for nitric oxide and from 2.07->200µg/ml for ferric chelation power.Phytochemical screening of leaves ethanol extract demonstrated the presence of alkaloids, phenol, terpenoids, tannins, steroids, flavonoids and glucosideswhile the HPLC profile revealed the presence of phenolic acids (o/p-coumaric acid; syringic acid) and flavonoids (vanillic acid). Overall, the results of this investigation demonstrated that the extracts from *Annona senegalensis* are potential sources of antioxidant metabolites needed to manage stress mediated disorders.

Keywords: *Annona senegalensis*, antioxidant activities, polyphenol contents, HPLC.

1. Introduction
Reactive oxygen species (ROS) are relatively unstable oxygen-bearing free radicals having a single unpaired electron in an outer orbit. They have been taught to be generated through exogenous factors such as tobacco smoke, ionizing radiation, and environmental pollutants as well as endogenous factors in nearly all eukaryotic cells during the course of normal metabolism, or disease development, and in response to tissue injury [1, 2].
In healthy individuals, the production of free radicals is balanced by the antioxidant defence system. They are formed at a reasonable concentration as necessary intermediates in a variety of normal biochemical reactions and are involved in defence mechanisms against pathogenic microorganisms and detoxification of harmful molecules [3, 4]. However, oxidative stress is generated when equilibrium between antioxidant and oxidant is broken and this lead to free radical or other reactive ROS generation as a result of a depletion of antioxidant levels [5, 2].
In almost all physio pathological conditions the amount of free radical produced is very high and the oxidative stress that results from this overproduction cause damage on vital biomolecule such as DNA, protein and lipid. In this condition, internal antioxidants become insufficient for balancing of free radicals so external antioxidants are needed to prevent the oxidative damages by directly reacting with ROS, scavenging them and/or chelating catalytic metal ions [6]. External antioxidants from natural origin are growing interest in preventing the destructive processes caused by oxidative stress. They are the alternative to synthetic antioxidants in counteracting the free radicals associated diseases [7] such as cancer, inflammation, Alzheimer and others [8, 9]. The interest in plants-based human health care emanates from their long use in folk medicines as well as their prophylactic properties, especially in developing countries. Plant extracts or their putative secondary metabolites have served as antioxidants in phytotherapeutic medicines to protect against various oxidative related diseases for centuries [10]. These trends have motivated the current tendency to replace synthetic phenols like Butylated hydroxyanisole (BHA), Butylated hydroxyl toluene (BHT) etc. with phenolic compounds extracted from natural sources with comparable antioxidant power and better safety attributes [11].
Nowadays, a large number of medicinal plants have been investigated for their antioxidant properties. Studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants such as, flavonoids, tannins, and proanthocyanidins. This chemical abundance of

medicinal plants may be responsible for their curative effect against several diseases [12]. Numerous reports have reveals positives and significant correlation between the polyphenol content of given plant extracts and their antioxidant properties [13, 14]. *A. senegalensis*is used in ethnomedicine against diarrhea, joints and respiratory diseases, conjunctivitis, wounds, snakebites, trypanosomiasis, jaundice, hemorrhoids, feminine barrenness, convulsions, ovarian cancer, fever, and asthenia[15]. Moreover, the phytochemical investigation of extracts from these Annonaceae species, widely distributed in Cameroon[16] has revealed a great diversity of phenolic compounds including epicatechin, catechin, rutin, isoquercetrin, anthocyanosides, tannins, as well as some saponosides, carotenoids, sterols, triterpenes, alkaloids, and cardiac glycoside, known for their antioxidant potential[17, 13, 14]. As mean to unveil the antioxidant profile as well as potent related bioactive phenolic, this study was designed to determinethe antioxidant and the phenolic content of ethanol extracts and fractions from *A. senegalensis.*

2. Materials and methods

2.1 Plant collection and extracts preparation

Leaves, twigs, and bark of *A. senegalensis* were collected at Balamba (Mbam and Inoubou Division) centre region of Cameroon, on the 25th April 2015 and identified at the National Herbarium of Cameroon where voucher specimens were deposited under the identification numbers 32071/HNC. Each plant part was cut into small pieces, dried at room temperature and ground to fine powder. One thousand grams of powders for each plant part were macerated in 95% ethanol for 72 hours at room temperature. The ethanol extracts were evaporated to dryness under vacuum using a rotary evaporator (RotavaporBuchi, Swizterland) and the solid residue was weighed. The crude extracts were subjected to liquid-liquid partition following the protocol described by Alali *et al*. [18]. The extraction yields were calculated as a ratio of plant extracts and plant powder. Crude extracts and fractions were stored at 4 °C until use.

2.2 Preparation of stock solution

Prior to each experiment, a stock solution of 1 mg/mL was prepared by dissolving the above prepared crude extracts and fraction in appropriated amount of 10% DMSO. From the stocks, serial dilutions were made to yield 25, 50, 100 and 200µg/mL. The total phenolic content as well as the antioxidant properties of extracts was assessed at 1 mg/mL.

2.3 Phytochemical screening of crude extracts

Phytochemical screening of extracts for the presence of alkaloids, anthocyanines, anthraquinons, glycosides, flavonoids, saponins, tannins, phenols, steroids and terpenoids was carried out following Harbone *et al*. [19] protocol.

2.4 Determination of the polyphenolic content by HPLC

Polyphenolic content was analysed by HPLC Waters 1525 system, fitted with a XTerra RP 18 column (4.6x 15mm 3.5um, Waters, USA). Samples were prepared in pure water at 1mg/ml and centrifuged at 10000 rpm for 10 min. The obtained supernatant was filtered through a cellulose acetate membrane filter (0.45 µm, Schleicher & Schuell). 100µL of extracts filtrate and standards (Gallic acid, Benzoic acid, Caffeic acid, Syringic acid, Vanillic acid, Apigenin, Catechin, Eugenol, O-Coumaric, P-Coumaric, O-tyrosol, Quercetin, Rutin, Tyrosol) were injected into the HPLC system. The column temperature was set at 40 °C. The mobile phase consisted of acetic acid at 1 % in pure water (solvent A) and 5% acetic acid in pure methanol (solvent B). The elution was performed following the gradient 0% to 100% of solvent B in 50minutes.

2.5 Antioxidant activity of crude extractsand fractions

The antioxidant activity of ethanol extracts and methanol fractions was investigated using the DPPH scavenging assay, the ferric reducing power assay and the nitric oxide (NO) radical scavenging assay.

2.5.1 DPPH radical scavenging assay

The radical-scavenging activity of crude extracts, fractions and standard antioxidant compound was measured using 1,1-diphenyl-2-picryl hydroxyl (DPPH). Briefly, 100µl of each fraction (dissolved in methanol or DMSO) was mixed with 900µl DPPH solution (dissolved in methanol). The mixture was homogenized, incubated for 1hat room temperature and reading was taken at 517 nm.The inhibition percentage was calculated using the following formula:

DPPH Scavenging activity (%) = (A-B)/A × 100

Where, A is the absorbance of control and B is the absorbance of sample at 517 nm.

2.5.2 Ferric reducing antioxidant power assay

The capacity of each plant extracts to reduce the ferric–ferry cyanide complex to the ferrous - ferry cyanide complex was determined as described by Batool *et al*. [20] with slight modifications. Briefly, 2.5 ml of different plant extract solutions were mixed with 2.5 ml of phosphate buffer (PH-6.6) and 2.5 ml of 1% potassium ferry cyanide. The mixture was incubated at 50°C for 20 min. After incubation 2.5 ml of 10% TCA was added to the mixture and centrifuged at 10000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then, the absorbance was measured at 510 nm by using UV spectrophotometer. Increment of the absorbance of the reaction mixture indicates increased reducing power. Similar method was adopted for Ascorbic acid used as a positive control. All the tests were performed in triplicates.

2.5.3 Nitric oxide (NO) radical scavenging assay

The nitric oxide radical scavenging assay was performedfollowing the procedure afforded by Panda *et al*. [21]. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of extracts (25, 50, 100 and 200µg/mL) and incubated at 25°C for 180 minutes. Extracts were mixed with an equal volume of freshly prepared Griess reagent. Control without extracts with an equal volume of buffer were prepared in a similar manner. The colour tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150 µL of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at 546 nm using a SpectraMaxPlus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the ethanol extracts and gallic acid were calculated using the following formula:

Nitrite oxide Scavenged (%) = (A-B)/A × 100
WhereA is the absorbance of control and B is the absorbance of sample or standard.

3. Results

3.1 Antioxidant results

DPPH scavenging activity of extracts

The dose-response curve of DPPH radical scavenging activity of crude ethanol extracts and methanol fractions of *A. senegalensis*, compared to gallic acid is shown in figure 1. It is observed that ethanol extract of leaves of *A.senegalensis* (AnsefeEtoH) exhibited higher radical scavenging activity as compared to the overall tested extracts. At 200µg/ml, a 60% scavenging ability activity could be registered with all tested extracts while at the same concentration, that of the gallic acid culminated at 94.85%. Moreover, the scavenging IC_{50} values represented in table 1 show the same tendency, by pointing out the ethanol extract from leaves (IC_{50}1.50µg/ml) of *A.senegalensis*, as the outstanding one followed by ethanol extract of twigs (IC_{50}4.50µg/ml) and bark (IC_{50}5.10µg/ml). Overall, ethanol extracts of all plant parts were found more potent than their methanol counterparts ($p \leq 0.05$).

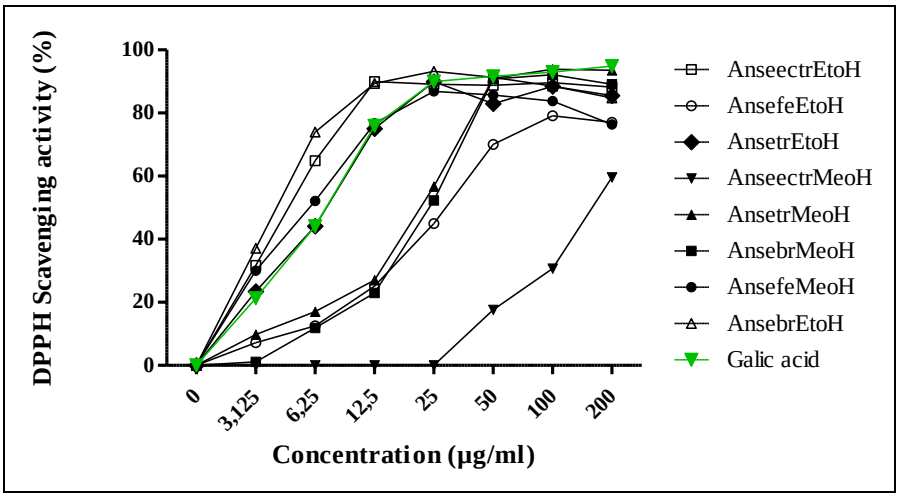

Fig 1: Free radical scavenging potential of *A. Senegalensis*extracts

3.2 Nitric oxide scavenging and ferric chelationpotentials of extracts

The nitric oxide activity of tested extracts was expressed by mean of IC_{50}. These ranged from 0.53->200µg/ml. The Least Significant Different (LSD) test revealed that ethanol extract of twig of *A. Senegalensis* with IC_{50} of 0.53 µg/ml was the most efficient followed by ethanol extract of leaves (IC_{50} 0.89 µg/ml) ($p \leq 0.05$).

3.3 Ferric chelation potentials of extracts

It's obvious from Table 1 that the tested extracts could successfully chelate ferric ions in reaction mixture. The chelation responses being variable from a given extract to another at 5% significance level. Overall, the chelation power ranged from 2.07->200µg/ml. Ethanol extract of leaves being once more the most active. Overall, methanol fractions were found very less potent than their respective crude extracts (*P*> 0.05).

Table 1: DPPH, Nitrite radical scavenging and Ferric chelating activities of *A. senegalensis* extracts.

Extracts	DPPH scavenging activity IC_{50} (µg/ml)	Nitricoxide scavenging activity IC_{50} (µg/ml)	Fe^{2+}chelationactivity IC_{50} (µg/ml)
AnsefeMeOH	6.40±1.13	1.23±0.001	>200
AnsebrMeOH	22.40±1.41	0.92±0.02	>200
Anse EctrMeOH	167.00±8.20	>200	>200
AnsefeEtOH	1.50±0.10	0.89±0.19	2.07±0.86
Anse Ectr EtOH	5.10±0.14	>200	>200
AnsebrEtOH	4.50±0.14	0.53±0.0007	49.51±0.41
galicacid	0.77±0.01	**	**

****not tested**

Phytochemical screening of crude extracts

Table 2: Phytochemicals screening of *Annona senegalensis* extracts.

Phytochemicals	Ansebr EtOH	Ansefe EtOH	AnseEctrEtOH
Alkaloids	-	+	-
Glucosides	+	+	+
Flavonoids	+	+	+
Steroids	-	+	-
Tannins	+	+	+
Saponins	-	-	-
Terpenoids	+	+	+
Phenol	+	+	+
Anthocyanins	-	-	-
Anthraquinons	- - -	-	-

Phytochemical screening of potent extracts revealed the presence of alkaloids, phenol, terpenoids, anthocyanins, saponins, tannins, steroids, flavonoids and glucosides.

3. HPLC analysis of extracts

The quantification of phenolic compounds content in plant extracts was done via calibration with standards. Results are expressed in mg/100g of dry sample.As outlined on HPLC profile exemplified in Fig. 2 and Table 2,ethanol extract from leaves of *A. senegalensis* demonstrated the presence of phenolic acids (o/p-coumaric acid; syringic acid, vanillic acid) and flavonoids (vanillic acid) at different concentration. Coumaric acid was found to be the most abundant in the tested extract.

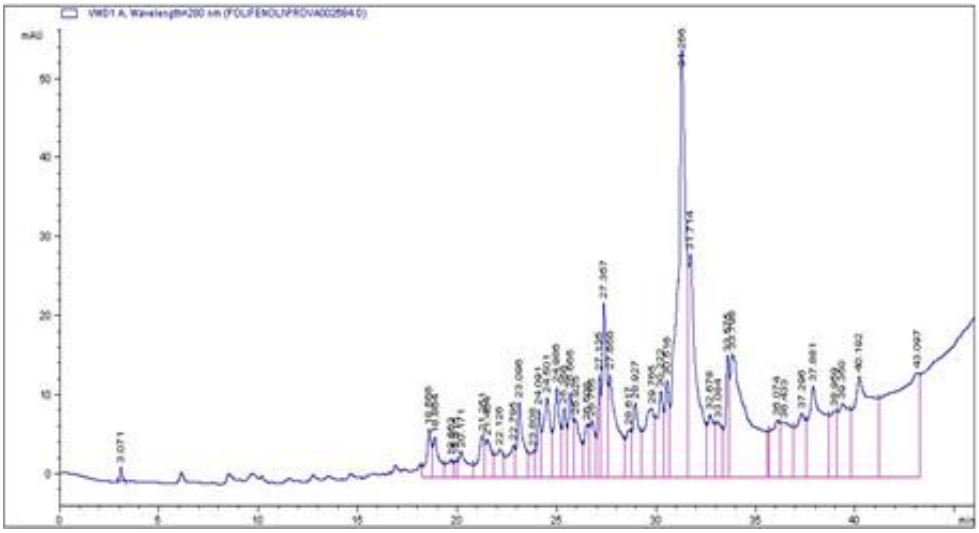

Fig 2: HPLC profile of extracts of AnsefeMeOH

Table 2: Amount of phenolic compounds in methanol fraction of leaves of *A. senegalensis*

Phenols standard		AnsefeMeOH	
Names	**Retention time (min)**	**Area (mUA)**	**Concentration (mg/g DW)**
Gallicacid	14.380	**	**
Benzoicacid	19.100	**	**
Caffeicacid	25.670	**	**
Syringicacid	25.550	163.677	4.058
Vanillicacid	25.270	131.111	4.188
Apigenin	33.490	**	**
Catechin	23.480	**	**
Eugenol	29.430	**	**
O-Coumaric	25.110	225.713	6.968
P-Coumaric	30.520	186.233	3.646
O-tyrosol	21.910	**	**
Quercetin	42.190	**	**
Rutin	29.450	**	**
Tyrosol	21.770	**	**

**not found

Discussion

Medicinal plants and other herbs which contain biological active compounds are widely used in food and cosmetic and drug industries, or as precursors in the synthesis of new products with specific bioactive properties [22]. *A. senegalensis*, a multipurpose medicinal plant found to exhibit several biological activities including antimicrobial can be an interesting candidate for phytomedicine formulation [15]. Therefore, this study was designed to investigate the antioxidant potential of ethanol extracts and methanol fractions using DPPH radical scavenging, nitric oxide scavenging and ferric chelation assay.

The screening of plant extracts using the DPPH free radical method have proved to be effective for the selection of those which could have an antioxidant activity[23]. The extracts involved in this study had revealed some scavenging potentials in function of the plant part. The ethanolic crude extract was the most efficient. These results go in the same line than that of Ajboye *et al* [24]. In fact, these authors had obtained an IC_{50} of 400µg/ml for DPPH scavenging activity from the aqueous extracts from leave of *A. senegalensis*. This difference in the results can be attributed to the extraction solvent. Furthermore, it can be suggested that the antioxidant compound from the leaves of this plants are non-polar as the activity were loose during fractionation. The phytochemical screening reveals the presence of Terpenoids, Polyphenols and Heterosides in all the plant parts. The alkaloids were detected only in the leaves extract. These results are close similar to that reported by Okoye *et al* [25] on methanol-methylene chloride extract from the roots bark of *A. senegalensis*.

The DPPH scavenging mechanism of extracts, thought to be due to their hydrogen donating ability is correlated with the presence of hydroxyl groups on the antioxidant molecule [26]. In the present study among all the extracts and fractions tested, ethanol extract of leaves (AnsefeEtOH) showed significantly higher inhibition percentage and positively correlated with total phenolic content. Nitric oxide is a potent pleiotropic mediator of various physiological processes. It is a diffusible free radical, which plays many roles as an effectors molecule in diverse biological systems [27]. Nitric oxide radical scavenging activity of various extracts of *A. senegalensis* was presented in table 1. All the extracts tested effectively reduced the generation of nitric oxide from sodium nitroprusside. AnsebrEtOH (IC_{50} 0.53µg/ml) showed highest nitric oxide radical inhibition followed by AnsefeEtOH (IC_{50} 0.89µg/ml) and AnsebrMeOH (IC_{50} 0.92µg/ml).

Iron is essential for life as it is required for oxygen transport, respiration and for activity of many enzymes. The ferric reducing power of extracts/compounds is determined by the decrease in the red color ferrozine-Fe^{2+} complex indicating high radical scavenging activity of the compound. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action [28]. The reducing ability of a compound

generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [29]. Overall, from this study, the tested extracts showed chelation power with IC_{50} ranged from 2.07->200µg/ml, Ethanol extract of leaves being the most active. This potent extract can therefore be used to inhibit lipid peroxidation by stabilizing the transition metals [30] and stabilized the oxidized form of the metal ion [31].

The above antioxidant potentials of ethanol extracts of leaves of *A. senegalensis* can relate to the phenolic content. In fact, the phytochemical screening and HPLC analysis revealed the presence of several phenolic compounds including phenol (o/p-coumaric acid; syringic acid), anthocyanins, tannins, and flavonoids (vanillic acid). Moreover, he has been reported that the antioxidant activity of plant extracts increased proportionally to the polyphenol content [32].

Conclusion

The results of this study suggest that the plant extract from *A. senegalens* contains phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

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