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Studies on phytochemical screening, total phenolic content and antiradical activity of three extracts of *Emilia sagittata* DC. (Asteraceae)

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Abstract

The present study determined the phytochemicals, antiradical activity, total phenolic and proanthocyanidin contents of three extracts of *Emilia sagittata* which was an herbaceous annual currently used to treat various diseases in gabonese pharmacopeia. The powdered plant samples were analyzed for their phytochemical screening using standard laboratory methods. The total phenols were measured using the Folin-Ciocalteu method. Proanthocyanidins content were determined by the method of HCl-butanol hydrolysis. Antioxidant activities were evaluated with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The best average contents of total phenols and proanthocyanidins were revealed among 423.80 mg GAE/10 mg extract and 57.11 mg APE/10 mg extract respectively, and an average of antioxidant capacity was found in 170.24 µg/ml ~~of~~ extract. Somewhat the proanthocyanidins in extract represent an average of 13.69 % for the total phenolic content. Also an average of 93% antioxidant capacity of extracts was due to the contribution of proanthocyanidins compounds thus are the dominant antioxidants in these extracts. The presence variability of bioactive extract compounds in the case of Emilia sagittata consolidated the fact of the traditional used of that plant in certain treatment diseases.

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Introduction

The last past years a major growing interest focus on substances from plant exhibiting antioxidant properties that could supplied animal organisms then human as food components or specific pharmaceutical activities were more and more analyzed (Azuma *et al.*, 1995). The antioxidant action was supposed to protect living organism from oxidative damages resulting in the prevention of various diseases. Plants should be the primary sources of naturally occurring antioxidants for humans.

Indeed, secondary metabolites wealth gives plants therapeutic properties that make them being the major source of drugs (Nostro *et al.*, 2000). Secondary metabolites are indeed important for our food (taste, color), while others such as alkaloids, anthocyanins, flavonoids, quinines, lignins, steroids, and terpenoids have been commercial application in the pharmaceutical and biomedical part of drugs, dyes, flavors, perfumes and insecticides.

The *Emilia* gathers could be a kind of plant belongs to the family Asteraceae from Africa, tropical Asia, and Oceanica. The specie *Emilia sagittata* DC is an herbaceous annual, weak-stemmed, to 1 m high, of roadsides and clearings of forest country, flowers solitary, terminal, dispersed from Guinea to Nigeria, Cameroons, and occurring through eastern Africa from Sudan to Mozambique, and into tropical Asia. It is an attractive plant of easy culture, bearing small scarlet or sometimes golden-yellow heads (Burkill, 1985).

Traditionally, the leaves are eaten fresh in salad or cooked as spinach in the region in Kenya. That plant is fed to rabbits and guinea-pigs in Gabon, and they eaten it with relish. The leaves are used to cover sores in Nigeria. A leaf-poultice mixed with copper filings is used in Gabon to dress ulcers. The sap is applied in Congo to ulcers, abscesses of the breast, leprous maculae and the ulcers of yaws, and also for mange, lice and ringworm. For the eyes, fresh sap is instilled in Nigeria and Gabon for soreness. In Gabon a leaf-macerate is taken for heart-affections (Raponda-Walker and Sillans, 1961; Burkill, 1985).

The purpose of this study is to report the phytochemical screening results, and total phenolic content details and analysis of antiradical activities from three extracts in the case of *Emilia sagittata* (Asteraceae) for contributing to the search for beneficial uses of this plant.

Materials and methods

Plant material

Emilia sagittata was collected at Melen I village, Bitam town, Gabon in September, 2012. The plant was first identified locally by its local name, aló-mvu or aki-küe and authenticated by a taxonomist at the Institut de Recherche en Ecologie Tropicale (IRET). The plant was then air-dried at room temperature.

Preparation of extracts for phytochemical screening

Air-dried powdered leaves (10 g) of *E. sagittata* were separately extracted with 100 ml (of each) of water (Aq), ethanol 50% (Et-H_2O) and ethanol 100% (EtOH) by maceration for 24 h. Extracts were filtered and the filtrate was used for photochemical screening.

Preparation of extracts for polyphenols measure and antioxidant activity

Air-dried powdered leaves (20 g) of *E. sagittata* were separately extracted with 150 ml (of each) of water (Aq), ethanol 50% (Et-H_2O) and ethanol 100% (EtOH) by maceration for 72 h. The choice of water was to mimic the traditional method of preparing crude plant extracts and that of ethanol was to enhance the extraction of more constituents. Extracts were filtered and dried under reduced pressure at 40° C. Aq extract (2.14 g, 10.73% of leaves dries), Et-H_2O extract (2.04 g, 10.2% of leaves dries), EtOH extract (1.10 g, 5.52% of leaves dries) were stored in freezer at 4°C until further tests.

Phytochemical screening

The three extracts, Aq, Et-H_2O and EtOH were screened for their classes of bioactive compounds using standard procedures (Culei, 1982; Harbone,

1984; Sofowora, 1993; Trease et Evans, 2002). The extracts were tested qualitatively for the presence of chemical constituents such as tannins, terpenes, saponins, flavonoids, cardiac glycosides, alkaloids, anthraquinones etc... For gallic tannins, 2 ml of 1% ferric chloride solution was added to 2 ml of the filtrate (Stiasny's test). Dark-greenish coloration indicated their presence. Tannins catechic, 2 ml of a solution of hydrochloric n-butanol are added to 2 ml of filtrate, and then heating in a water bath for 5 to 10 minutes (Bate-Smith's test). Intense red coloration indicated the presence of the catechin tannins. For total flavonoids and anthocyanes, 1 ml of the sulfuric acid was added to 2 ml of the filtrate, then 1 ml NaOH. There shown a dark color after adding acid, indicating the presence of flavonoids, the color changes to purple after addition of NaOH, indicating the presence of anthocyanins. 2 ml of the filtrate were added magnesium strips followed by hydrochloric alcoholic (cyanidine test). A rose-orange effervescence showed the presence of flavones, rose-purplish indicated of flavanones and red denoted of flavonols. We had applied the Folin's test to determine polyphenols contents. 1 ml of the Folin reagent was added to 2 ml of the filtrate, then 1 ml NaOH. Dark green coloration indicated the presence of polyphenols. For coumarins, 2 ml of filtrate combined with 2 ml of NH_4OH, then, lookin at UV lamp (366 nm). The fluorescence presence indicated the presence of coumarins. 2 ml NH_4OH solution was added to 2 ml of the filtrate (Borntrager's test). A rose pink colour in the ammonia layer indicated the presence of anthraquinones. For alkaloids, some drops of sulfuric Dragendorff's reagent were added to 2 ml of the filtrate. Orange precipitate formed had showed the presence of alkaloids. To determine cardiac glycosides and terpenes, test such as Salkowski's and Lieberman's test were applied. 2 ml of concentrated H_2SO_4 were added to 2 ml of filtrate, a reddish-brown ring indicated the presence of steroid, an aglycone part of the cardiac glycoside (Salkowski's test). Another part of the filtrate (2 ml) was added with 2 ml of acetic anhydride and cooled well in ice and concentrated H_2SO_4 (2 ml) was carefully added. A colour change from blue to green indicated the presence of terpenes (Lieberman's test). Saponins were determined through frothing test. The filtrate was vigorously shaken. Frothing which persisted on warming for about 15 min indicated the presence of saponins. For reducing sugars, equal volume of Fehling's A and Fehling's B reagents were taken in equal quantities and were added to filtrate and boiled on water bath (Fehling's test). Appearance of brick red precipate indicates the presence of reducing sugars. Cardiac glycosides: to 1 ml of filtrate 1 ml of ferric sulfate solution (5 %) and 2 ml of concentrated sulfuric acid gives a color reaction Kiliani-Keller based structure cardiac glycosides into play, namely (Parekh *et al.*, 2006): Digitoxin: dirty red brown - Digitoxigenin: red fluorescent - Gitoxin: yellow then red blue - Gitoxigenine: yellow then red purple.

Determination of total polyphenol and proanthocyanidin contents

The Folin-Ciocalteu method was used to measure total amount of polyphenol content (Singleton *et al.*, 1999). Aliquots of 0.25 ml of leaf extracts (1 mg/ml) were mixed with 1.25 ml Folin–Ciocalteu reagent (0.2 N diluted in MeOH). A reagent blank using MeOH instead of sample was prepared. After 5 min incubation at room temperature, 1 ml sodium carbonate solution (7.5%) was added. Samples were incubated at room temperature for 1 h and the absorbance was measured at 765 nm versus the prepared blank. All tests were carried out in duplicate and polyphenol content was expressed as mg of gallic acid equivalents (GAE) per 10 mg of leaf extract.

Proanthocyanidins (PAs) were quantified with the hydrolysis test of proanthocyanidins in a hot acid-alcohol medium into anthocyanidins. This method allows taking into account all the units of flavans-3-ols constituting the polymers (Prigent, 2005). The heating step destroys the anthocyanidins pigments generated by flavan-4-ols and eliminates part of the chlorophyll pigments. The routine assay was performed by mixing 0.16 ml (1 mg/ml) of the

extract with 2.33 ml of 30% HCl-butanol solution (v/v). The mixture was put in tightly closed tube and vortexed for 1 min. Subsequently, the tube was heated at 100°C for 2 h and after cooling, the absorbances were read at 550 nm. Apple procyanidins (DP ≈ 7.4) treated as aforementioned were used as a standard. Results were expressed as apple procyanidins equivalent (APE).

Radical-scavenging activity assay

Scavenging activity of 2, 2-diphenyl-1-picylhydrazyl (DPPH) radicals of extracts were measured according to the Blois (1958) with minor modifications. Various concentrations of sample extracts (1 ml) were mixed with 1 ml of methanolic solution containing DPPH radicals (20 mg/l). After 15 min incubation at room temperature in the dark, absorbance of the reaction mixtures was measured at 517 nm. The inhibitory effect of DPPH was calculated according to the following formula: $[(A_{control}-A_{sample}) / A_{control}]*100$. The radical scavenging activity of extracts of leaf was expressed as the IC_{50} value (µg/ml), i.e., the concentration necessary to decrease the DPPH concentration by 50 %.

Statistical analysis

Experimental results were expressed as mean ± standard deviation. All measurements were replicated three times. The data were correlated using Pearson correlation coefficient at $p < 0.05$. The IC_{50} values were calculated using linear regression analysis from the graph of scavenging effect percentage against extract concentration.

Results and discussion

Phytochemical screening

The phytochemical screening of the extracts was first performed to detect the major chemical groups occurring in the extracts. The results of this screening were shown in table 1. The qualitative analyze of extracts had shown the presence of saponins, tannins, alkaloids, triterpenoids, flavones, anthraquinones, coumarins, proanthocyanes, digitoxine (cardiac glycoside) and reducing sugars in the *Emilia sagittata*. There was a slight difference in the phytochemical constituent of the three extracts. However, among the extracts, the aqueous one was the extracts that contained the most detected chemical groups. The screening of chemical extracts of *E. sagittata* had shown that they were rich in phenolic compounds. Phytochemically, the plant samples were quite rich, containing tannins, alkaloids, Reducing sugars, triterpenoids, polyphenols in substantial quantities in all extracts. Saponins were absent in Et-H_2O and EtOH extracts but abundant in aqueous extract. Coumarins and digitoxine were present in high concentration in Et-H_2O extract, abundant in aqueous extract and rare in EtOH extract. The flavones were very abundant, abundant and rare in aqueous, ethanolic and hydro-ethanolic extracs, respectively. We observe the rare presence of anthraquinones and proanthocyanes only aqueous extract.

Commonly, phytochemicals were responsible for various bioactivities such as antimutagenic, anticarcinogenic, antioxidant, antimicrobial and anti-inflammatory properties etc (Bruneton, 2009). Therefore, the richness of its extracts active chemical compounds could explain the traditional use of *Emilia sagittata* to cure many diseases such as: cutaneous, subcutaneous parasitic infection; eye treatments; leprosy; paralysis, epilepsy, convulsions, spasm, mucosae; stomach troubles; yaws, venereal diseases, heart; pulmonary trouble etc. as fabrifuges; generally healing; as laxative; skin diseases (Burkill, 1985). Indeed, several authors had been shown that different types of chemical compounds had identified in the extracts of this plant with therapeutic effects (Bruneton, 2009). For example, the alkaloids were the most efficient plant substances used therapeutically. Pure isolated alkaloids and the synthetic derivatives were used as the basic medicinal agent because of their analgesic, antispasmodic and bacterial properties. This explain why the *E. sagittata* leaf was believed to stop asthma, convulsions, spasm; stomach troubles and was prescribed for cutaneous, subcutaneous parasitic infection cure (Burkill, 1985). Similarly presence of

glycosides and tannins had shown the hypoglycemic potential of the plant (Cherian and Augustin, 1995).

Table 1. Results of phytochemical screening of extracts from *Emilia sagittata*.

Chemical constituents		Aqueous extract	Ethanolic-water extract	Ethanolic extract
Saponins		++	-	-
Tannins	Gallic	+++	+++	+++
	Catechin	++	++	+++
Alkaloids		+++	+++	+++
Triterpenoids		+++	+++	+++
Polyphenols		+++	+++	+++
Flavonoids	Flavonols	-	-	-
	Flavones	+++	+	++
	Flavanones	-	-	-
Free anthraquinones		+	-	-
Coumarine		++	+++	+
Proanthocyane		+	-	-
Cardiac glycosides	Digitoxine	++	+++	+
	Digitoxigenine	-	-	-
	Gitoxine	-	-	-
	Gitoxigenine	-	-	-
Reducing sugars		+++	+++	+++

Legend: -: Not detected, +: Rare, ++: Abundant, +++: Very abundant.

This phytochemicals and the significant presence of reducing sugar had shown also dietary values of the plant (Oloyed, 2005); although, some of these analyzed constituents could be completely harmful to man. Some of these active components (tannins…) had been demonstrated to possess anti nutritional effects, following their ability to reduce palatability and digestibility of feedstuff (Odebiyi and Sofowora, 1979).

Total phenolic and proanthocyanidin Content

Levels of phenolic content were expressed in terms of gallic acid equivalent (GAE). The equation of the righthand side of the proportioning of total phenolic content by the method of Folin-Ciocalteu gave Y = 0.0012 X – 0.0004 with R^2 = 0.9902 (Abdoul-latif *et al.*, 2012). The total contents of phenols ranged between 223.25±0.01 and 581.58±0.15 mg GAE/10 mg of leaf extract (Table 2). The three extracts were not showed significant differences in their content in total phenols (P>0.05). The aqueous extract had showed maximum phenolic content followed by ethanol 50% extract with 581.58±0.15 and 466.58±0.16 mg GAE/10 mg, respectively. Ethanolic extract had showed minimum phenolic content of 223.25±0.01 mg GAE/10 mg. The phytochemical screening of the extracts had revealed that the main phenols in *E. sagittata* were tannins. Flavones were much present only in the aqueous extract. Phenolic substances have been suggested to play a preventive role in the development of chronic diseases such as cancer and heart disease (Njintang *et al.*, 2012).

The HCl/butanol assay used here for the determination of proanthocyanidins is more specific than many other tests such as the vanillin assay. The interferences, which might result from flavan-4-ols conversion into proanthocyanidins or from chlorophylls, should have been minimized during the heating step (Prigent, 2005; Santos-Buelga and Scalbert, 2000). Levels of proanthocyanidins were expressed in terms of apple procyanidins equivalent (APE). The equation of the right-hand side of the proportioning of the proanthocyanidins by the HCl-Butanol method gave Y = 0.0006 X + 0.0024 with R^2 = 0.9869 (Abdoul-latif *et al.*, 2012). Among extracts, proanthocyanidin contents had ranged between 33.5±0.01 and 81.83±0.03 mg APE/10 mg of extract (Table 2). The aqueous extract had contained the highest amount of proanthocyanidins followed by ethanolic 50% extract (81.83±0.03 and56±0.03 mg APE/10 mg of extract, respectively) and the ethanolic extract had revealed weak proanthocyanidins content (33.5 ± 0.01 mg APE/10 mg of extract).

Thus, proanthocyanidins in *E. sagittata* extracts represent on average 13.69 % of total phenolic content. There were not a significant variation of the content of proanthocyanidins among leaf extracts.

Table 2. Comparison of total phenolic compounds, proanthocyanidins and antioxidant capacity of *Emilia sagittata* leaf extracts.

Extracts	Total phenols (mg GAE/10 mg of extract)	PAs (mg APE/10 mg of extract)	Quota of PAs in Total phenols (%)	DPPH: IC_{50} (µg/ml)
Aqueous extract	581.58 ± 0.15	81.83 ± 0.03	14.07	173.35 ± 29.08
Ethanolic-water extract	466.58 ± 0.16	56 ± 0.03	12	169.26 ± 24.88
Ethanolic extract	223.25 ± 0.01	33.5 ± 0.01	15	168.12 ± 29.21

Antiradical activity

The free radical-scavenging activities of various extracts were evaluated at their initial concentration. All extracts showed free radical scavenging activity (Table 2). Lower IC_{50} indicating the higher antiradical activity of the extract. The crude ethanolic extract had best DPPH free radical-scavenging activities (IC_{50} value = 168.12±29.21 µg/ml) followed by ethanolic-water extract (IC_{50} value = 169.26±24.88 µg/ml). Aqueous extract which had high total phenols contents, exhibited weak antiradical activity (IC_{50} value of 173.35±29.08 µg/ml). The antiradical activity not differed significantly in the extracts ($P > 0.05$). This results were unexpected, indeed, it was expected that aqueous extract with high levels of total phenols, could exhibit higher antiradical activity. This was not the case since ethanolic extract had the lowest total phenol content (223.25 mg GAE/10 mg), now the total phenols content has widely been shown to correlate positively with antioxidant activity (Figure 1). The opposite results observed here probably highlighted the differences in the phenolic profiles, some being more active than others. That suggested that the radical scavenging capacity of each extract might be mostly related to their concentration of phenolic hydroxyl group. The antiradical activity of phenolic compounds depends on their molecular structure: on number, on position, on nature of the substituent on the rings B and C (hydroxyl, glycosylated…) and the degree of polymerization, on the availability of phenolic hydrogens, and on the possibility for stabilization of the resulting phenoxyl radicals ($AO^{\bullet}$) formed by hydrogen donation (Olajire and Azeez, 2011).

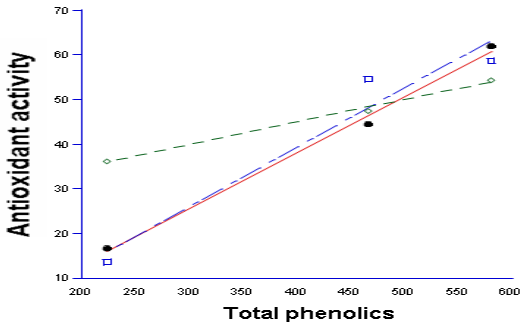

Fig. 1. Correlation between antioxidant activity (inhibitory effect of DPPH) of the three extracts and total phenolics. ● (—) = antioxidant activity of Aq extract, (R^2 = 0.9901); □ (— – —) = antioxidant activity of Et-H_2O extract, (R^2 = 0.9124); ◇ (~ ~ ~) = antioxidant activity of EtOH extract, (R^2 = 0.8667)

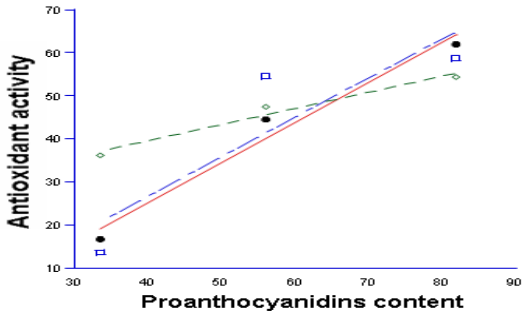

Fig. 2. Correlation between antioxidant activity (inhibitory effect of DPPH) of the three extracts and proanthocyanidins content. ● (—) = antioxidant activity of Aq extract, (R^2 = 0.9833); □ (— – —) = antioxidant activity of Et-H_2O extract, (R^2 = 0.8188); ◇ (~ ~ ~) = antioxidant activity of EtOH extract, (R^2 = 0.9815)

On the other hand, this difference is likely not significant that the dosage by Folin-Ciocalteu reagent is not specific to polyphenols, but many compounds such as proteins, reducing sugars, ascorbic acid and sulfur compounds can react with this reagent, giving

an apparent high rate phenolic (Singleton *et al*., 1999; Tawaha *et al*., 2007). Water is more polar than ethanol; it is more likely not significant to extract with a large number of polar compounds such as sugars.

The correlation coefficient between the content of the extracts of E. sagittata proanthocyanidins and antioxidant activity was highly significant (R^2 = 0.9833, R^2 = 0.8188, R^2 = 0.9815 for the aqueous extracts, ethanolic and aqueous-ethanolic, respectively), indicating that on average 93% of the antioxidant capacity of extracts is due to the contribution of compounds proanthocyanidins and antioxidants that are dominant in these extracts. These results are consistent with the results of Park *et al* (2011), who reported a positive correlation between such proanthocynidine content and antioxidant activity. The results of the determination of the antioxidant capacity of extracts depend greatly on the methodology used. It appeared that it is important to compare different analytical methods varying in their oxidation initiators and targets in order to understand the biological capacity of an antioxidant (Cao and Prior, 1999). The antioxidant capacity could depend on the sample, response time and type of phenolic compounds, which means that different phenolic compounds react in different ways.

Conclusion

Our study showed that the analysis of the *Emilia sagittata* extracts were performed to determine levels of phytochemicals content, total phenolic content, proanthocyanidins and antioxidant capacities. The chemical substances present in this plant highlight a beneficial antioxidant capacity of the *E. sagittata* against the free radicals *in vitro*. Thus, this plant could be considered as good sources of antioxidant, and has been shown according to their IC_{50} with potential anti-cancer and antiathesclerosis properties. Also their total phenolic and proanthocyanidins contents had been determinated. A significant correlation was obtained between antioxidant activity and total phenolic content as reciprocally the same phenomenon between antioxidant activity and proanthocyanidins content than indicating that phenolic compounds contribute significantly to antioxidant activity of the investigated extracts, mainly the proanthocyanidins content. Finally the present experiment suggests that proanthocyanidins could provide the interest for being use as alternative antioxidants.

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