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Antioxidant Activity of Aqueous Methanol and Ethyl Acetate Extract of Leaves of *Annona senegalensis* Pers from Togo Versus the One Originates from Burkina Faso

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Abstract: The aim of the present study is to evaluate and compare the antioxidant potential of the leaves extracts of *Annona senegalensis* (Annonaceae) of Togo versus the one of Burkina Faso. To this end, aqueous methanol and ethyl acetate extracts by splitting and by steeping were achieved and the determination of total polyphenols of which flavonoids was carried out. A survey of the antioxidant activity using the DPPH methods was performed. The content in total polyphenol (3.47±0.03%) and flavonoid (2.33±0.17%) of 70% (v/v) aqueous methanol extract of the specimen from Togo was significantly higher than the one from Burkina (2.66±0.08 and 1.64±0.04%, respectively) ($p<0.00001$ for total polyphenol; $p<0.05$ for total flavonoid), whereas, the amount of total flavonoid in the ethyl acetate extract of the species from Burkina (40.38%) was triplicated. For the two types of extracts, the species of Burkina Faso showed an improved antioxidant activity than the one of Togo (IC_{50} = 8.51 and 21.08 μg mL^{-1} versus 12.46 and 29.22 μg mL^{-1}, respectively) ($p<0.05$). These free radicals inhibition activity of the extracts may be due at least to polyphenolic flavonoids identified by means of HPLC assay performed in the preliminary study. These flavonoids were rutin and isoquercetrin as flavanols (specimen from Togo) of which are added epicatechin and catechin derivatives (flavanols) in the specimen from Burkina. The traditional use of plant leaves may imply in part this activity against the free radicals.

Keys words: *Annona senegalensis* from Togo, *Annona senegalensis* from Burkina Faso, aqueous methanol extracts, ethyl acetate extracts, total polyphenols, total flavonoids, antioxidant activity

INTRODUCTION

The free radicals are chemical species ($O_2^{\bullet -}$, $OH^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, H_2O_2, O_2*) (Coudert *et al*., 1994) produced in the organism during the chemical reactions that contribute to the development and the maintenance of the cellular life. These are very reactive species towards endogenous molecules (DNA, proteins, lipids) having a beneficial role (antimicrobial activity) but implied especially in the pathological physiology of numerous affections: atherosclerosis, heart failure, ageing, neurodegenerative disorders, cancer, diabetes mellitus and a plethora of other diseases (Coudert *et al*., 1994; Abdi and Ali, 1999). So, one constantly attends nowadays the increasing development of substances having the suitability to stop the initiation and/or the propagation of reactions (oxidization) leading up to the production of these free radicals: These are the free radical scavenging or antioxidant agents which are synthesis substances but more and more the phytochemical principles of medicinal plants.

In the preliminary phytochemical study, we have also reported the circumstances of choice of *Annona senegalensis* as subject of study; we have described its physical characteristics, its ecosystem of growing and its numerous traditional uses not only in Togo but also in many other countries of Africa.

A review of the literature showed that the free radical scavenging activity has not yet been evaluated nor in the leaves nor in any other part of the plant. In order to value the antioxidant potential of this plant, our work intends to do this compared to the species originates from Burkina Faso using extracts from the leaves.

MATERIALS AND METHODS

This study has been carried out during the year 2006 at the Institute of Research in Science of Health (IRSS/CNRST), Department of Medicine and Traditional Pharmacopeia, Ouagadougou, Burkina Faso.

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Collection of the plant material, extraction and phytochemical screening of the extracts: To this end, a preliminary study has previously been performed.

Determination of total polyphenolic compounds: The concentration of total polyphenol compounds in the 70% (v/v) aqueous methanol extract (Ehm) were determined spectrophotometrically on the basis of a standard curve ($R^2 = 0.9988$) plotted using tannic acid (serial dilutions to give a range of 3.33-23.33 μg mL^{-1}). Briefly, 50 μL of extract and the standard previously dissolved in 60% methanol were diluted (3-fold replicated). The absorbance of the studied extract and the serial concentrations of the standard were measured with an UV-Visible Agilent spectrophotometer 8453 E at 280 nm against a blank.

The content in total polyphenolic compounds was calculated using the equation of the standard curve and the results expressed as mg of Tannic Acid Equivalent (TAE) per 100 mg of lyophilised extract.

Determination of total flavonoid compounds: The total flavonoids (a class of polyphenols) content of 70% aqueous methanol extract and ethyl acetate extract (Eea) was determined with the help of Dowd method adapted by Arvouet-Grand *et al.* (1994) using 2% aluminium trichloride in methanol as reagent. The absorbance was measured using an Agilent UV-Visible spectrophotometer 8453 E at 415 nm against a blank. The standard curve ($R^2 = 0.9986$) was plotted using the serial concentrations of quercetin.

The content in total flavonoid constituents was calculated using the equation of the standard curve and the results expressed as mg of Quercetin Equivalent (QE) per 100 mg of lyophilised extract.

Evaluation of the antioxidant activity

Qualitative determination of the antioxidant activity: It is a rapid test in order to evaluate the antioxidant activity on the precipitated fraction of ethyl acetate extracts of both samples. Based on the method previously described (Burits and Bucar, 2002), the solutions of each extract and quercetin as standard were spotted on the starting point on silica gel F254 of a thin layer chromatography sheet and developed in an appropriate migration solvent system. After this, the silica gel sheet is allowed to dry and sprayed with 4% 2, 2-diphenyl-1-picryl-hydrazil (DPPH, a purple coloured free radical generator) in methanol. Any bleaching of the purple color background of DPPH reagent to the site of the spots within 30 min was taken as positive result.

Quantitative determination of the antioxidant activity: The free radical scavenging potency of 70% aqueous methanol and ethyl acetate extracts was determined using the method of Mensor *et al.* (2001) with some modifications. Briefly, each extract and quercetin (21 mg mL^{-1}), as positive control, were diluted to give concentrations ranging 1050-0.5 μg mL^{-1} with dimethyl sulfoxide (DMSO). The solution of 4% DPPH in methanol was prepared. Two hundred microliter of this solution was added to 10 μL of each extract or quercetin solutions in various concentrations and allowed to react in the steam room at 37°C for 30 mn. The blank and the negative control were prepared with appropriate solvents. Each assay was 4 fold replicated. The decrease in absorbance (deep violet to light yellow) of the mixtures of tested extracts or quercetin was measured at 490 mn on a spectrophotometer Biorad Model 680 Microplate Reader. These absorbance values were converted to the percentage of inhibition of free radical scavenging activity (I%) using the formula:

$$I\% = \frac{A_O - A_S}{A_O} \times 100$$

where, A_s was the absorbance of the sample, A_o was the absorbance of the control. The IC_{50} (concentration causing 50% inhibition of a maximum effect estimate in 100%) value of each extract was calculated using the equation of linear regression of plots (excel software) of concentrations of tested samples (μg mL^{-1}). The inhibition of DPPH activity of quercetin was also measured under the same condition to serve as standard antioxidant agent.

RESULTS

Phytochemical contents of the extracts: The phytochemical principles identified in the preliminary study were mainly polyphenols (flavonoids, catechol tannins, anthocyanosides), saponosides and carotenoids for the 70% aqueous methanol extract and anthocyanosides, flavonoids and carotenoids for ethyl acetate extract. The High Performance Liquid Chromatography (HPLC) analysis of the extracts has confirmed the presence of flavonoids, especially rutin and isoquercetrin (flavonols) in the specimen from Togo and the preceding compounds plus epicatechin and catechin derivatives (flavanols) in the specimen from Burkina.

Determination of total polyphenolic and flavonoid content: Table 1 shows the content in total polyphenols and flavonoids (a group of polyphenol) of Ehm and Eea from

Table 1: Total polyphenolic and flavonoid content and the antioxidant activity (IC_{50}) of 70% aqueous methanol and ethyl acetate extract of plant material from Togo versus the one originates from Burkina Faso

Extracts	Total polyphenolic content (mg±SEM TAE/100 mg dry extract)	Total flavonoid content (mg±SEM QE/100 mg dry extract)	IC_{50}±SEM (µg mL^{-1})
Ehm Tg	3.47±0.03[a]	2.33±0.17[c]	12.46±1.05[g]
Ehm Bf	2.66±0.08[b]	1.64±0.04[d]	8.51±0.66[h]
Eea Tg	-	13.27±0.20[e]	29.22±2.03[i]
Eea Bf	-	40.38±1.08[f]	21.08±0.31[j]
Quercetin	-	-	2.99±0.21[s]

Comparison of total polyphenolic and flavonoid content (Student's t-test): a vs b ($p<0.00001$), c vs d ($p<0.05$), e vs f ($p<0.0001$), Comparison of IC_{50} values (Student's t-test): g vs s ($p<0.001$), h vs s ($p<0.001$), i vs s ($p<0.0001$), j vs s ($p<10^{-8}$), g vs h ($p<0.05$), i vs j ($p<0.05$)

both plants. The quantitative composition of Ehm was 3.47±0.03% of total polyphenols and 2.33±0.17% of total flavonoids for *Annona senegalensis* of Togo (AsTg) versus, respectively 2.66±0.08% and 1.64±0.04% for *Annona senegalensis* from Burkina Faso (AsBf).

For Eae, the content in total polyphenols of which mainly flavonoids was 13.27±0.20% in Togolese sample versus 40.38±1.08% in the plant originates from Burkina Faso.

Comparative antioxidant activity of the extracts
Qualitative determination of the antioxidant activity: This chromatogram photography (Fig. 1) shows the positive test of DPPH bleaching to the site of spots issued from ethyl acetate extract of sample originates from Togo (Eea Tg) versus the one from Burkina Faso (Eea Bf) and Quercetin (Q) as standard.

Quantitative determination of the antioxidant activity: Table 1 shows the antioxidant potential of Ehm and Eea expressed as the IC_{50} value in comparison to the positive control (quercetin). For both plants, 70% aqueous methanol extract exhibited a highest antioxidant activity (IC_{50} = 8.51±0.66 µg mL^{-1} for AsBf versus 12.46±1.05 µg mL^{-1} for As Tg) than ethyl acetate extract (IC_{50} = 21.08±.31 µg mL^{-1} for AsBf versus 29.22±2.03 µg mL^{-1} for AsTg).

The two extracts from *Annona senegalensis* of Burkina Faso showed a potent antioxidant activity (IC_{50} = 8.51±0.66 µg mL^{-1} for Ehm versus 21.08±0.31 µg mL^{-1} for Eea) compared to those from Togo (IC_{50} = 12.46±1.05 µg mL^{-1} for Ehm versus 29.22±2.03 µg mL^{-1} for Eea).

Statistical analysis: The results of total polyphenolic and flavonoid content, the IC_{50} values of the antioxidant activity are expressed as mean±SEM of three or five determinations. The determination of total polyphenolic and flavonoid content were issued from the

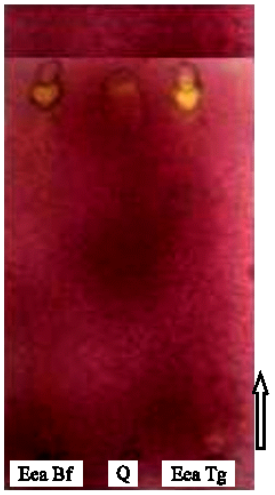

Fig. 1: Chromatogram of ethyl acetate extracts of Eae Tg versus Eae Bf and quercetin (Q) as standard. The arrow indicates the migration steering of the solvent

concentration-absorbance curves. The IC_{50} values were obtained from the dose-response regression plots. In the two cases, good coefficients of correlation R^2 were observed: 0.9986 and 0.9643 to 0.9842, respectively. A low IC_{50} value is an indication of a strong antioxidant activity. The two samples were compared in regard to the quantitative determination and the antioxidant activity using the Student's t-test. A probability P of 0.05 or less was considered significant.

DISCUSSION

The determination of total polyphenols and its related group flavonoids showed that the content of these constituents is significantly higher in Ehm Tg (3.47±0.03 and 2.33±0.17%, respectively) than in Ehm Bf (2.66±0.08 and 1.64±0.04%, respectively) ($p<0.00001$ for total polyphenol; $p<0.05$ for total flavonoid). Concerning Eea, as reported by Ibewuike *et al*. (1997) and Aderogba *et al*. (2003, 2004, 2005), the main polyphenolic group that have been isolated in ethyl acetate extract of leaves were flavonoids. In this study, the highest amount (40.38±1.08%) of flavonoids were found in ethyl acetate extract of the specimen collected at Burkina Faso ($p<0.0001$) (Table 1). These flavonoids are represented at least by rutin, isoquercetrin, epicatechin and catechin derivatives as has detected HPLC assay.

These quantitative and qualitative differences noted in the extract content of phytochemical principles of both species may be explained, as we have discussed in a previous phytochemical screening study, by the climatic

conditions (Pieters and Vlietinck, 2005; Reynolds, 2002; Metting and Pyne, 2007), the geological environment (Gomes and Silva, 2007) of the sites of harvest, the period of the collect, the enzymatic content responsible for the biosynthesis pathways (Pieters and Vlietinck, 2005) and the regulation of gene expression by environmental factors (Boudet, 2007). Indeed, if one considers that the substances elaborated by the plant allow it to adapt to its environment (role of signal, defense against the predators and the parasites, resistance against the harmful bugs and the diseases) (Pieters and Vlietinck, 2005), the one of Burkina being more arid (Sub-Saharan climate), this difference can be explained.

The two extracts of both samples belonging to two different ecosystems exhibited a substantial but a least antioxidant activity (IC_{50} = 8.51-29.22 μg mL^{-1}) in relation to the quercetin (IC_{50} = 2.99 μg mL^{-1}) ($p<0.001$). The DPPH inhibition activity of aqueous methanol extracts (IC_{50} = 8.51 μg mL^{-1} for AsBf versus 12.46 μg mL^{-1} for AsTg) was more potent compared to the one of the ethyl acetate extracts (IC_{50} = 21.08 μg mL^{-1} for AsBf versus 29.22 μg mL^{-1} for AsTg) ($p<0.0001$). Indeed, the antioxidant activity of the plant extracts depends on: the type and the polarity of the extracting solvent, the extracting technique, the purity of the active principle, the antioxidant test, the substrate used (Tsuda *et al*., 1994) and the structural requirements (a number of phenolic hydroxyl groups on ring structures) (Harborne, 1986; Saskia *et al*., 1996; de Beck *et al*., 2003).

Although the content in total polyphenols and their related flavonoids was higher in Ehm Tg ($p<0.00001$ for total polyphenol; $p<0.05$ for total flavonoid), the Ehm Bf has showed the best scavenging activity with IC_{50} value of 8.51 μg mL^{-1} versus IC_{50} value of 12.46 μg mL^{-1} for Ehm Tg ($p<0.05$). Relating to the ethyl acetate extracts, the total flavonoids content of Eea Bf was 3-fold higher than Eae Tg (40.38±1.08% versus 13.27±0.20%) ($p<0.0001$). However, this Eea Bf exhibited a weak scavenging potential compared to the one of Ehm Bf (21.08 versus 8.51 μg mL^{-1}) but more potent than the one of Eae Tg (IC_{50} = 21.08 μg mL^{-1} versus 29.22 μg mL^{-1}) ($p<0.05$).

The effectiveness of the antioxidant activity of the extracts of *Annona senegalensis* from Burkina may be explained by the environmental factors such as the climatic conditions, the stage of plant maturation, the temperature (Gazzani *et al*., 1998). McCune and Johns (2005) reported that this antioxidant activity was increased under growing conditions of decreased water and fertility, what is the case of the Sub-saharan climate like the growing ecosystem of *Annona senegalensis* originates from Burkina. Besides, a big diversity exists in the efficiency of the antioxidant activity of different phenolic compounds (Montalleb *et al*., 2005). The antioxidant activity reported in this study is related in part to the previous flavonoids (rutin, isoquercetrin, epicatechin and catechin derivatives) which involve at least a catechol group necessary to radical scavenging in any flavonoid that possesses this activity (Saskia *et al*., 1996; de Beck *et al*., 2003).

Despite the highest amount of total flavonoids of Eea Bf (40.38±1.08%), this one didn't exhibited a highest scavenging activity than Ehm Bf which contained only 1.64±0.04%. This finding confirms the data of the literature according to which no any relationship exist between the antioxidant activity and the content of total flavonoids (Galvez *et al*., 2005; Sawadogo *et al*., 2006), even though their free radical scavenging activity is not anymore to demonstrate.

The free radical scavenging activity of the 70% aqueous methanol and the ethyl acetate extracts of *Annona senegalensis* leaves is due in part to their phytochemical constituents of polyphenols of which the flavonoids as reported numerous authors (Pieters and Vlietinck, 2005; Galvez *et al*., 2005; Aderogba *et al*., 2004, 2005); other polyphenols like the tannins (Pieters and Vlietinck, 2005), the anthocyanins (Wang *et al*., 1999) and the saponins (Alaoui *et al*., 1998) as terpenoid identified in the extracts could also participate to this antioxidant activity. Indeed, these chemical principles have been reported to be involved in the antioxidant activity of various medicinal plants.

The present study showed that the leaves extracts of *Annona senegalensis* from the two ecosystems, especially the one of Burkina Faso constitutes a potent natural source of antioxidant agents. This antioxidant activity could justify in part, the use of this medicinal plant in traditional practice.

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