ANTIOXIDANT ACTIVITY AND EXTRACTABILITY OF PHENOLIC COMPOUNDS FROM MEDICINAL PLANTS: A STUDY OF *GLYPHAEA BREVIS* SPRENG. (MONACH.)

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Summary

This study was carried out to assess the possible utilization of *Glyphaea brevis* as a source of antioxidant phenolics and to compare different extraction processes of these compounds. Leaves and extracts of *Glyphaea brevis* were analyzed for antioxidant activity using three methods: ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS). The phenolic content was also determined. Multiple-stage extraction processes with different solvents were used to extract *Glyphaea brevis* and subsequently compared to obtain the most suitable way to maximize phenolics extraction. Phenolic contents of *Glyphaea brevis* products correlated highly with their antioxidant activity measured by the three methods ($r \geq 0.90$). A two-stage extraction process using water and/or ethanol was found to be a low-cost and high-profit way to obtain phenolics from *Glyphaea brevis*. The results obtained suggest the possibility of *Glyphaea brevis* being used as a source of antioxidant phenolics. This may explain its use in traditional medicine in various areas and could find applications in the effective management of oxidative stress and related degenerative diseases.

Keywords: *Glyphaea brevis*, ROS, oxidative stress, antioxidant, phenolics, extractability, FRAP, DPPH, ABTS.

Introduction

A little fraction of the oxygen we consume daily through respiration leads to the formation of oxidative products and reactive oxygen species (ROS) in the body. The main sites of ROS production in the respiratory chain are the enzymatic complex I and the proton motive Q cycle operating in the complex III (1).

This natural process is not the only source of ROS since some environmental pollutants are also involved in their generation. ROS promote oxidation in the cell (pro-oxidants) and, if not neutralized, may trigger several diseases, attacks of tissues (lungs, heart, kidneys, liver, gut, eyes, skin, muscles and brain) and ageing. Fortunately, endogenous antioxidants such as glutathione and antioxidant enzymes (Mn^{2+}- dependent superoxide dismutase (MnSOD), copper/zinc SOD, glutathione peroxidase (GPx), glutathione reductase (GR) and catalase, CAT) in the organism prevent damages caused by ROS. These antioxidants catalyze the conversion of ROS into hydrogen peroxide that is further transformed into water under the action of catalase (2).

However, during some illnesses, infanthood or ageing, the process of ROS neutralization may not function properly. A variety of pathologies, including cancer, diabetes, ischemia, inflammatory diseases, the aging process, and cardiovascular disease, have been linked to the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($OH^{\bullet}$) (3) through the high oxidative imbalance (oxidative stress) they induce in the cell. Therefore, the intake of exogenous antioxidants derived from food and other sources may be beneficial to prevent lipid peroxidation, protein cross-linking and DNA mutations among others (4).

Phenolic compounds including flavonoids, lignoids and tannins are secondary metabolites distributed in the plant kingdom. Their structures characterized by multiple labile protons enable them to scavenge ROS, thus preventing subsequent oxidative damage. Many of their beneficial effects in animal and human organisms are reported such as anti-aging, anti-inflammation (5), anti-carcinogenic (6,7), anti-mutagenic (8), anti-ulcer (9), anti-atherogenic effects and as the inhibitors of human low density lipoprotein oxidation (10,11,12). They constitute a highly available and low-cost source of antioxidants. It is therefore appropriate to carry out research on new plant sources of phenolics and to maximize the yield of the extraction of these valuable compounds.

Glyphaea brevis Spreng.(Monach.), a Tiliaceae, is widely distributed in Africa and South America. It is valued there as vegetable (13) and various therapeutic uses such as treatment of hepatitis and poisoning are reported (14). Recent work also highlighted its anticonvulsant properties (15). Therapeutic activities of various medicinal plants have been sometimes related to their antioxidant properties (16). Therefore, antioxidant activity could be accountable for the curative properties of *Glyphaea brevis* through a contribution to redox homeostasis.

The aim of this study is to investigate the possibility of *Glyphaea brevis* Spreng.(Monach.) as a potential source of antioxidants by assessing its phenolic content, the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) free radicals scavenging activity, the ferric reducing antioxidant power (FRAP). We also compare different multiple-stage extraction processes to maximize the extraction of phenolics from *Glyphaea brevis*.

Methods

The leaves of *Glyphaea brevis* Spreng. (Monach.) were harvested in the city of Douala (Cameroon) during March 2006. The taxonomy was confirmed by Mr. Nana, Cameroon National Herbarium, Yaounde, Cameroon (voucher specimen no.10781/SRF/Cam).

Leaves were dried to constant weight in an oven at 40°C for 48 h. After that, they were ground into a fine powder (250 *μ*m mesh sieve) that was kept in opaque plastic flasks at 25°C and used 2 days later for the study.

Samples for measurement of free antioxidant capacity (non hydrolyzed samples) were prepared as described by Agbor *et al* (17). 100 mg of leaf powder were introduced in a centrifuge tube and 10 ml of water were added. The tube was hermetically closed and placed in a warming bath (95°C) for 90 min with 3-second shaking every 30 min. After cooling, the tube was centrifuged at 2000 *g* for 10 min. The supernatant was collected and stored at – 20°C for analytical studies. The same process was applied to samples that were to be used for measurement of total antioxidant capacity (hydrolyzed samples), except that distilled water was replaced by a methanolic solution of hydrochloric acid 1.2 N (16).

All the samples were screened for phenolic content while only non hydrolyzed samples were used to assess antiradical activity. In all the assays, catechin was used as standard. Phenolic content of the samples was measured using the Folin-Ciocalteu method (18). The ferric-reducing antioxidant power (FRAP) of the samples was assessed as described earlier by Benzie and Strain (19). DPPH antiradical activity of the samples was measured as described by Katalinié *et al* (20). ABTS antiradical activity was assessed as described by Re *et al* (21).

Conventionally, some organic solvents and water have been used to extract phenolics from plant materials (22,23,24,25). Five solvents were used to extract phenolics from the leaves of *G. brevis*: water, ethanol, ethyl acetate, chloroform and hexane. Because of the viscosity of the powder leaf when in contact with liquids, we realized different powder-liquid mixtures with different masses of dry matter in different volumes of solvent. Finally, the formulation made of 3.75 g of leaf powder in 50 ml of solvent was retained. The mixture was allowed to macerate at room temperature for 48 h and centrifuged at 2000 *g* for 5 min. The supernatant was collected and the solid residue was thrice further extracted under the same conditions as for the first extraction. Moreover, a decoction was prepared with water. This was done by boiling the powder-water mixture for 15 min. Supernatants from each extraction were conserved at – 20°C for the analysis of the phenolic content.

All the abovementioned assays were performed in replicates of four and the results presented as mean ± standard deviation. Duncan's test was used for multiple comparisons of the yields of different multiple-stage extractions and the antioxidant activities. The relation between the methods was established by applying Pearson product moment correlation. The software SPSS for Windows (SPSS Inc., Chicago, IL, USA) version 10.1 was used for the analysis. P-values less than 0.05 were considered as significant.

Results

Phenolic content and antioxidant activity of *G. brevis*

Table 1 shows the amounts of free and total phenolic compounds contained in a mass unit of dry leaves and the antioxidant activities determined by different methods. *G. brevis* presented a phenolic content varying from 20.80 ± 1.96 mg CE/g (non hydrolyzed samples) to 59.86 ± 1.63 mg CE/g (hydrolyzed samples) (Figure 1). In all cases, the total phenolic contents were 1.5 – 3 times higher than the free phenolic contents. The antioxidant activities determined by the ABTS and DPPH methods were comparable and inferior to those obtained with the FRAP method. Phenolic contents of the samples correlated strongly with their antioxidant activity measured by all three methods. (Table 2).

Table 1. Comparison of antioxidant activities of leaves and extracts of *G. brevis*

		Phenolics	FRAP	ABTS	DPPH
Leaves	Free	20.80 ± 1.96^{a}	5.73 ± 0.38^{a}	5.38 ± 0.12^{a}	5.20 ± 0.65^{a}
	Total	59.86 ± 1.63^{b}	20.05 ± 1.11^{b}	-	-
Aqueous extract	Free	39.29 ± 1.63^{c}	16.95 ± 0.43^{c}	8.03 ± 0.13^{b}	9.41 ± 1.29^{b}
	Total	79.16 ± 0.82^{d}	25.82 ± 0.88^{d}	-	-
Hydroalcoolic extract	Free	81.47 ± 2.45^{d}	20.04 ± 0.29^{b}	12.48 ± 0.20^{c}	16.47 ± 0.83^{c}
	Total	106.90 ± 0.82^{c}	38.34 ± 0.46^{c}	-	-

All the values are expressed in milligrams catechin equivalents per gram (mg EC/g).
In each column, values not sharing the same superscript letter differ significantly at P<0.05 (Duncan).

Table 2. Correlation coefficients between the antioxidant activities measured by different methods

	Phenolics	FRAP	ABTS	DPPH
Phenolics	-	0.93	0.90	0.96
FRAP	-	-	0.77	0.96
ABTS	-	-	-	0.86

Only statistically significant (p<0.01) values are shown in the table.

Phenolics extractability from *G. brevis*

Quantities of phenolic compounds extracted as a function of the solvent used and the number of extractions performed are presented in Figure 1. The highest quantities of total phenolics extracted were obtained with aqueous and ethanolic solvents, while hexane and chloroform gave the lowest yields. Figure 1 also shows that the majority (75 – 100%) of phenolics extracted are removed during first and second extractions with decreasing amounts removed in subsequent extractions.

There was no significant difference between the total yields of the four-stage extractions using maceration or decoction. However, there were some differences in the quantities extracted at each stage. For example, the first stage of the aqueous extraction by decoction was more efficient than the two first stages of the aqueous extraction by maceration with 20.39 ± 0.43 mg CE/g vs 17.32 ± 1.25 mg CE/g ($P<0.01$).

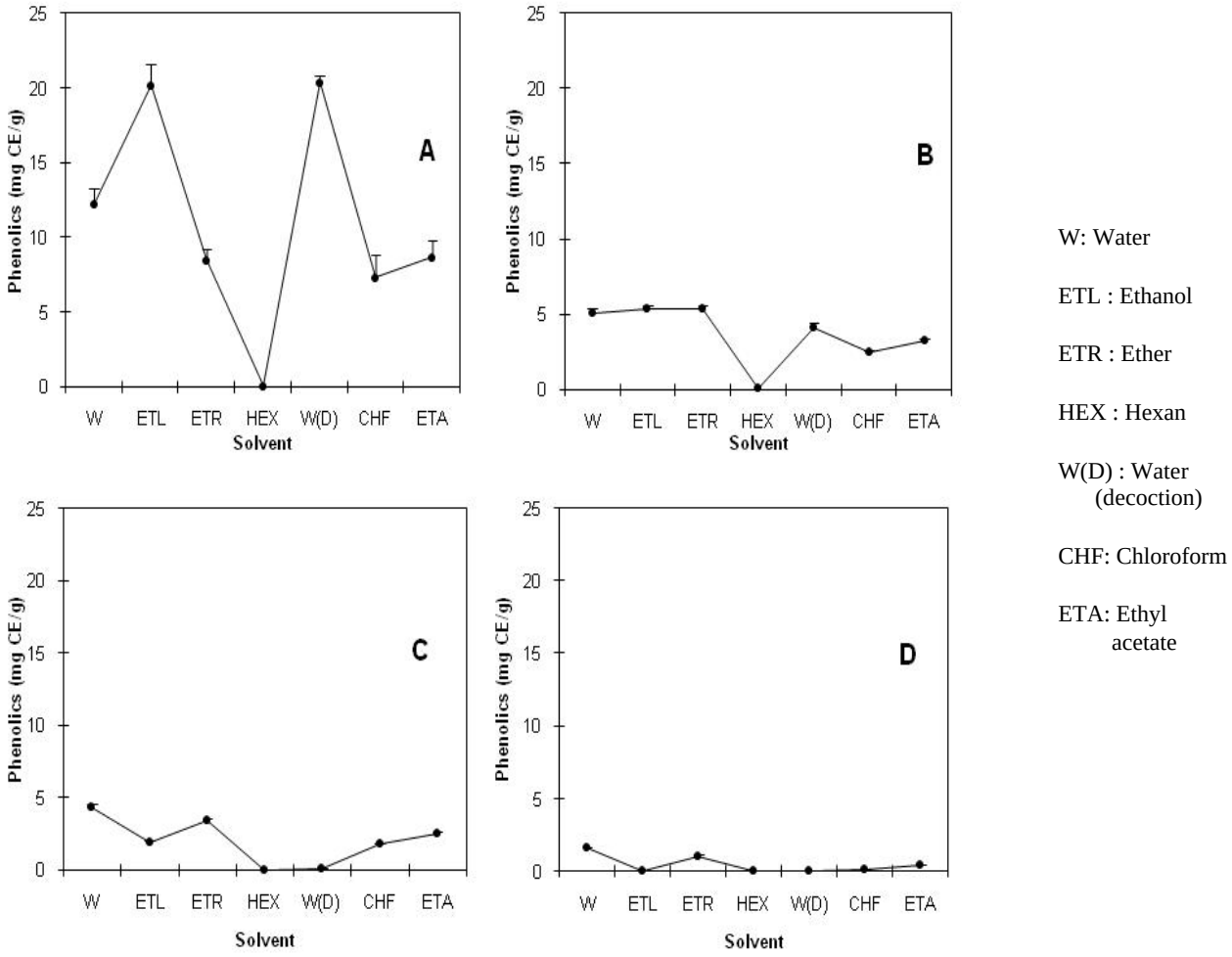

Figure 1: Comparison of various four-stage extractions of phenolics from *Glyphaea brevis* using different solvents. A) First extraction ; B) Second extraction ; C) Third extraction ; D) Fourth extraction. Quantities are in milligrams catechin equivalent per gram of dry weight (mg CE/g). (*n*=4)

Discussion

As a comparison, it is known for several years that grape seeds usually contain 5000 – 8000 mg/kg total phenolics depending on the variety of grape (26). *G. brevis* also presented an antioxidant activity, as determined by FRAP, ABTS and DPPH methods.

The highest yields obtained with polar solvents are due to the polar structure of phenolics. Figure 1 also shows that the majority (75 – 100%) of phenolics extracted are removed during first and second extractions with decreasing amounts removed in subsequent extractions. From an industrial production point of view, numerous extractions of the same sample of plant material would mean waste of solvent and lower efficiency of equipment utilization. Particularly in the case of aqueous extraction (by decoction) and ethanolic extraction, two stages could extract 93% to 99% of obtained phenolics. Therefore it is not cost efficient to add one more stage for the low quantity of remaining phenolics (0.15 – 7%).

The cost of time and energy inputs for a third stage and removal of the liquid added may be higher than the value of phenolics extracted in this stage. Safety concerns associated with the use of organic solvents such as ethyl acetate for industrial extractions include solvent residues in the product, exposure to workers and disposal of waste solvents and pollution of the environment (25). A two-stage extraction process using water or ethanol would represent a safer and more cost efficient technique to obtain phenolics from plant materials.

One stage of aqueous extraction by decoction was more effective than two aqueous extractions by maceration (20.386 ± 0.432 mg CE/g vs. 17.320 ± 1.246 mg CE/g) suggesting that hot water is more efficient than cold water. This difference in phenolics extracted observed between aqueous extraction by maceration at room temperature and by decoction is an illustration of the effect of solvent temperature on the yield of extraction. This is also the case of tea polyphenols. In a recent study (27), heating at 90°C for 10 minutes significantly increased the extraction yield of tea polyphenols, compared to extraction at lower temperatures. Shi *et al.* (25) advanced the hypothesis that heating might soften the plant tissue and weaken the phenol-protein and phenol-polysaccharide interactions in plant material meal, thus allowing more phenolics to migrate into the solvent. Higher temperatures of solvent appear to be a means to reduce the time necessary to run the extraction process. We also assessed the effect of ethanol concentration on hydroalcoholic extraction of phenolics. We performed two-stage extractions with different concentrations of ethanol (20, 30, 40, 50, 60, 70, 80 and 95 %, volume / volume). The highest quantity of phenolics extracted was observed when using the hydroalcoholic solvent containing 50% ethanol (Data not shown). This suggests that phenolic compounds contained in leaves of *G. brevis* are made of molecules that are soluble in water and others that are soluble in organic solvents. This differential solubility could be related to the nature of the molecules that are esterifying or etherifying the phenolics. We also decided, based on the extractability study, to run a two-stage extraction process followed by evaporation to obtain solid aqueous (AE) and hydroalcoholic (HAE) extracts. Phenolic content and antioxidant capacity of both extracts were determined as described earlier (Table 1) and their free radical inhibition profile was determined by assessing antiradical activity as a function of extract concentration.

Inhibition percentages were obtained for each extract concentration through the formula: Inhibition (%) = $[(A_0 – A) / A_0] \times 100$ were A_0 is the absorbance in absence of extract and A the absorbance in presence of extract. Both aqueous (AE) and hydroalcoholic (HAE) extracts presented inhibiting effects on the free radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (Table 3).

In general, values of extract concentrations inhibiting by 50% the free radicals (IC_{50}) were 10 – 20 times higher with $DPPH^{\bullet}$ radical than with $ABTS^{\bullet+}$. This suggests a differential reactivity of the phenolics of *G. brevis* with each of these radicals. The speed of reaction in the methods may also be a factor explaining these differences. In a study on sorghum and sorghum extracts, Awika *et al.* (28) reported that samples reacted rapidly with $ABTS^{\bullet+}$ while they reacted slowly with $DPPH^{\bullet}$ and deducted from this that the reactivity of the antioxidants in sorghums with these free radicals is somehow slowed in alcoholic media. This could explain the very low reactivity of aqueous extract (AE) toward $DPPH^{\bullet}$ since the assay must be done in alcoholic media (methanol or ethanol) while ABTS does not have such a constraint because of its solubility in both aqueous and organic solvents. In both antiradical assays, hydroalcoholic extract appeared as having a better inhibiting activity than aqueous extract, probably due to their respective phenolic contents.

Table 3. Inhibition profiles of free radicals ABTS and DPPH by extracts of *G. brevis*

Product	ABTS			DPPH		
	Equation	R^2	IC_{50} (mg/ml)	Equation	R^2	IC_{50} (mg/ml)
Aqueous extract	Y= 14.33 lnX + 92.45	0.87	0.05	Y= 63.59X +14.61	0.96	3,01
Hydroalcoholic extract	Y= 11.70 lnX + 94.01	0.91	0.02	Y= 13.8X + 8.44	0.93	0,56

Y= percentage inhibition; X= extract concentration

The higher concentration of phenolics in hydrolyzed samples is due to the fact that phenolic compounds in food occur mainly in their conjugate form, that is esterified or etherified, and only partially in the free form. For instance, phenylpropanoids which occur predominantly in grains and cereals are often esterified while flavonoids which are dominant in fruits occur as glycosides. In this relation, biological activity of compounds involved might be different from those examined in *in vitro* systems. Hence, this suggests the necessity to subject phenolics to hydrolysis prior to their evaluation.

FRAP, ABTS and DPPH values were lower than the phenolic content (Table 1). This would be due to the fact that antioxidant activity of phenolics depends on their structure and the number of hydroxyl groups it may contain (29).

The strong correlations observed between phenolic contents and antioxidant activities suggest that phenolics are largely accountable for the antioxidant activity of *G. brevis* and its extracts. Several authors have reported similar correlations between phenolics and antioxidant activity measured by various methods (11,30,31,32). Therefore the phenol content of a given sample of *G. brevis* can be considered as a relative predictor of its antioxidant activity.

DPPH values were in general slightly higher than ABTS values and with the only significant difference for HAE ($P<0.05$). However, individual antioxidant molecules are more efficient at quenching certain radicals than others (33). Therefore, the relative rank in activity of different samples across methods is more relevant than absolute values for comparing activities (28).

A significant correlation was observed between ABTS and DPPH ($r= 0.86$, $P<0.05$) demonstrating that all the samples had comparable activities in the two systems (Figure 10).

FRAP values higher than ABTS and DPPH values could be explained by the fact that FRAP assay estimates only the Fe (III) reducing activity, which is not necessarily relevant to antioxidant activity physiologically and mechanistically. This reduction is achieved through the transfer of both electrons and hydrogen atoms while in the case of antiradical methods, the quenching of free radicals is mainly assured by hydrogen atoms transfer (33). From this point of view, FRAP would appear to be a sum of the antioxidant power derived from antiradical activity plus that derived from electronic reduction of iron and may lead to overestimation of the real antioxidant activity. Therefore, estimation of antioxidant power of a given extract should not limit to this only method. Antiradical methods should be performed as better indicators of antioxidant activity of plant materials, based on the reaction mechanisms involved (33). Ou *et al.* (33) noted that pH and color interference may also be involved in these differences.

In this study, the results of the different antioxidant methods applied to *G. brevis* and *G. brevis* extracts showed that they can actually constitute a source of antioxidants through their phenolic content. The extractability of the phenolics contained in *G. brevis* was influenced by the solvent used, its temperature and the number of extraction stages. Addition of ethanol to water improved the extraction efficiency and reached a maximum at the concentration of 50% ethanol in water. Heating increased the concentration of phenolics in the aqueous extraction due to more phenolics extracted from *G. brevis* leaves. A two-stage extraction process using water or ethanol appeared to be opportune to maximize the yield of phenolics extraction. These results confirm the antioxidant potential of medicinal plants and may explain the use of *Glyphaea brevis* in traditional medicine in various areas. This could find applications in the effective management of oxidative stress and related degenerative diseases through further *in vivo* studies.

Acknowledgements

The authors gratefully acknowledge the comments of Dr Bertille C. Tchankou and Mr. Martin A. Mune Mune. Help of Dr Jean Michel Onana, National Herbarium of Cameroon, is highly appreciated.

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