



Exploration of the Chemical Potential and Antioxidant Activity of Some Plants Used in the Treatment of Male Infertility in Southern Benin

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JRK and VTD designed the study and performed the statistical analysis. Authors EA and MY wrote the protocol and wrote the first draft of the manuscript. Authors TIS and EA managed the analyses of the study. Author ED managed the literature searches. Authors JTD and JMA was ensured the correction and finalization of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Antioxidants are a family of substances that can neutralize free radicals and prevent and/or treat diseases associated with oxidative stress such as male's infertility. Medicinal plants are one of the main sources of antioxidants.

Aim: This work was aimed at evaluating the chemical and antioxidant potential of different extracts from some plants used in traditional Beninese medicine for the treatment of male infertility.

Materials and Methods: The study was carried out on aqueous, hydro-ethanolic and ethanolic extracts from the roots of *Gardenia ternifolia* (*G. ternifolia*), the whole plant of *Cassythia filiformis* (*C. filiformis*), the leaves of *Rourea coccinea* (*R. coccinea*) and the seed of *Garcinia kola* (*G. kola*). Quantification of the total polyphenols and flavonoids content of these extracts was evaluated respectively by the method using Folin Ciocalteu and the method using Aluminum trichloride. The antioxidant activity of the extracts was evaluated by molecular spectrophotometry using the free radical scavenging of DPPH and FRAP methods.

Results: The results obtained indicated a variation of total polyphenols and flavonoids content according to the type of extract. Hydro-ethanolic extract of the various plants studied has a high polyphenols and flavonoids content. In variable proportions, all the extracts tested reduced the DPPH radical and ferric iron, reflecting their antioxidant potential. The best antioxidant activity has been obtained with the hydro-ethanolic extracts.

Conclusion: This study showed that all the plant's extracts studied have antioxidant activity that varies with the type of extract. However, the hydro-ethanolic extractions showed the best antioxidant activities. The data obtained in the present study justified the use of these plants in management of pathologies involving oxidative stress.

Keywords: Antioxidant activity; medicinal plant; male's infertility; Benin.

1. INTRODUCTION

Oxidation is an essential process in the metabolism of aerobic cells of the body. It involves the oxygen molecule whose production by uncontrolled metabolic pathways causes the formation of reactive oxygen species (ROS) [1]. The free radicals are involved in oxidative stress which is characterized by an imbalance between the production of (ROS) (pro-oxidants) and their elimination by the antioxidant defence mechanism [2]. It has been described as a crucial etiological factor involved in various chronic human diseases such as cancer, cardiovascular and neurodegenerative diseases, diabetes mellitus, infertility and ageing [3]. Oxidative stress (OS) causes oxidative damage resulting from the free radical attack of various biomolecules, particularly proteins, lipids and DNA, and ultimately causes cell degradation and apoptosis [4]. To escape these consequences of the deleterious (OS), it is necessary to restore the oxidant/antioxidant balance to preserve the physiological performances of the organism [5]. Antioxidants are a family of substances that can neutralize free radicals and thus prevent and/or treat diseases associated with oxidant stress. They act as a major defence against ROS-induced toxicity by protecting the cell membrane

and cytosolic compounds. Synthetic antioxidants, as well as conventional products endowed with antioxidant potentials, are particularly known for their effectiveness. However, their use is increasingly being questioned because of their potential toxicological risks [6]. In this context, new sources of natural antioxidants are being actively sought [7]. Medicinal plants, known as providers of bioactive substances, are a major source of antioxidant molecules [8]. Several pharmacological investigations have shown the antioxidant potential of different plant extracts and have indicated a variation in antioxidant power depending on the plant and the type of extract [1,6,9].

Benin has an interesting ethnobotanical potential. The mission of Akoègninou et al. [10] identified 2807 plant species. *R. coccinea*, *G. ternifolia*, *G. kola*, and *C. filiformis* are four plants of the Beninese pharmacopoeia frequently used in the treatment of pathologies associated with oxidant stress as male infertility. Despite these uses in traditional Beninese medicine, very little pharmacological data exists at the present stage on the antioxidant potential of these plants. This study aimed at evaluating the chemical potential and antioxidant activity of various extracts of *R. coccinea*, *G. ternifolia*, *G. kola* and *C. filiformis*.

2. MATERIALS AND METHODS

2.1 Study Material

Plant Materials consists of vegetable samples of *G. ternifolia*, *C. filiformis*, *R. coccinea* and *G. kola*. These vegetable species were selected after an ethnopharmacological survey. Samples of *G. ternifolia*, *C. filiformis* and *R. coccinea* were harvested while that of *G. Kola* was purchased in southern Benin. Their botanical identification was carried out at the National Herbarium of Benin at the University of Abomey-Calavi. Table 1 presents the information relating to the four selected plant species, their place of harvest, the month and year of harvest.

Reagents and solvents used in this study include distilled water, Ethanol, Vitamin C, DPPH (2,2-Diphenyl-1-picrylhydrazyl), BHT (Butyl Hydroxy Toluene), Rutin, Folin Ciocalteu reagent, Gallic acid, Iron chloride ($FeCl_3$), $AlCl_3$ etc. Reagents obtained near the Research Unit in Applied Microbiology and Pharmacology of Natural Substances (URMAPha) of the University of Abomey Calavi (UAC-Benin) was purchased from Medical Services Benin Sarl (Proformat Invoice No. 041/MSB-19).

2.2 Methods of Study

2.2.1 Collecting plant samples and production of extracts

Plant organs were collected in southern Benin in Bohicon, Djidja and Zakpota cities in January and March 2019. After their identification at National Herbarium of Benin, organs were dried in the laboratory at a temperature of 16°C for 14 days. The dried material has been powdered using a Retsch SM 2000/1430/Upm/Smf type an electric grinder. These powders were then used for the aqueous, hydro-ethanolic and ethanolic extractions of each plant species by the methodology described by Fah et al. [11]. Fifty (50) grams of powder was macerated in 500 mL of solvent (water, ethanol and water-ethanol mixture of equal volume). The mixtures were left

stirring continuously for 72 hours at room temperature. The homogenate obtained has been filtered three times on hydrophilic cotton and once on Wittman No. 1 paper. The filtrate obtained has been evaporated at a temperature of 40°C in an oven (oven) until a dry mass that represents the extract. The extract obtained has been weighed and used to evaluate the extraction yield (EY) and then kept in the refrigerator at 4°C.

$$EY = \frac{\text{Mass of the extract after evaporation of the solvent}}{\text{Mass of the powder of the plant species used for extraction}} \times 100$$

[12]

2.2.2 Determination of bioactive molecules

2.2.2.1 Determination of total polyphenols content

The total phenols were assayed by a method adapted from that of Singleton et al. [13] using the commercial Folin Ciocalteu Reagent (FCR). In short, 50 µL of the extract was mixed with 250 µL of the FCR (10 times diluted in distilled water) and 750 µL of an aqueous solution of sodium carbonate Na_2CO_3 (7.5%). After 8 min of incubation, 950 µL of distilled water was added and mixed with the vortex and incubated for 2h. Optical densities (OD) were read at 760 nm using a CECIL CE 2041 spectrophotometer. The reading was made against a blank consisting of a mixture of 250 µL of FCR, 750 µL of Na_2CO_3 and 1 mL of distilled water. The total polyphenol content in the various extracts was calculated from a linear calibration curve ($y = ax + b$), established with precise gallic acid concentrations as a reference standard (0-200 µg/mL). The total phenolic content was determined as mg of gallic acid equivalent/g of extract (mg GAE/g) by using formula Ahmed et al. [14]:

TPC = $(X \times V)/m$; where TPC is total phenolic content, X is the gallic acid concentration in mg/ml; V is the extract volume used in ml and m is the weight of extract in grams.

Table 1. Information related to the medicinal plants selected

Vegetable species	Botanic family	Parts of plant used	Collection site	Month of harvest	Year of harvest	Voucher number
<i>C. filiformis</i>	Lauraceae	Whole plant	Djidja	January	2019	YH 262/HNB
<i>G. kola</i>	Clusiaceae	Seed	Bohicon/Market	January	2019	YH 260/HNB
<i>G. ternifolia</i>	Rubiaceae	Roots	Djidja	January	2019	YH 263/HNB
<i>R. coccinea</i>	Connaraceae	Leaves	Zakpota	March	2019	YH 261/HNB

2.2.2.2 Determination of total flavonoids content

The contents of the flavonoids were measured by a suitable method of Zhishen et al. [15] and Kim et al. [16] using aluminium trichloride (AlCl₃) as a reagent. Briefly, 500 µL of AlCl₃ (2%), 500 µL of the extract and 3 mL of methanol were mixed thoroughly. The blank consists of 500 µL of AlCl₃ and 3.5 mL of methanol. Absorbance reading is done at the spectrophotometer at 415 nm after 10 min of incubation. Samples were prepared in triplicates for each analysis and the mean values were taken. The quantities of flavonoids in the extracts were calculated from the calibration curve of a standard flavonoid (Rutin) as a reference standard (0-1 mg/mL). The total flavonoids content was determined as µg of rutin equivalent/g of extract (µgRuE/g) by using the formula Ahmed et al. [14]

TFC = (X x V)/m; where TFC is total flavonoids content, X is the rutin concentration in mg/ml; V is the extract volume used in ml and m is the weight of extract in grams.

2.2.3 Antioxidant activity of plant extracts

2.2.3.1 Trapping the radicals of DPPH

The method adopted in this study is that of Agbangnan et al. [17]. A volume of 100 µL of different concentrations of each extract added to 1900 µL of the ethanolic solution of DPPH (0.4 mg/mL). The white prepared by mixing 100 µL of the extraction solvent with 1900 µL of the DPPH solution. After incubation in darkness for 1 hour at room temperature, the reading of the absorbances was performed at 517 nm using a MINDRAY spectrophotometer (BA-88-A). These measured absorbances were used to calculate the percentage of trapping of the DPPH radical that is proportional to the antioxidant power of the sample. Vitamin C and BHT were used as reference standards. Antioxidant activity is expressed as the percentage of trapping determined by the formula:

$$P = \frac{AW - AS}{AW} \times 100$$

P: Percent trapping, AW: absorption of the white; AS Absorption of the sample.

2.2.3.2 Iron reduction test (ferric reducing-antioxidant power)

The reducing power of the extracts was determined by the FRAP method (Ferric

Reducing Antioxidant Power) according to the protocol described Dieng et al. [18] 0.5 mL of the sample at different concentrations is mixed with 1 mL phosphate buffer (0.2 M; pH= 6.6) and 1 mL potassium hexacyanoferrate [K₃Fe(CN)₆] at 1%. After incubation at 50°C for 30 minutes, 1mL of 10% trichloroacetic acid was added and the tubes were centrifuged at 3000 rpm for 10 minutes. Then 1 ml of the supernatant from each tube is mixed with 0.2 mL of FeCl₃ solution at 0.1% and allowed to stand under light for 30 minutes before measuring the absorbances at 700 nm. Vitamin C and BHT used as reference standards. The antioxidant activity related to the reducing power of the extracts is determined by the following formula:

$$PR = 100 (AS - Ab) / AS$$

PR: Reducing power, AS: Absorption of the sample, AW: Absorption of the white.

2.3 Statistical Analysis of Data

The data obtained were subjected to statistical analysis using SPSS 16.0 and Graph Pad Prism 7. Quantitative variables were presented as mean and standard deviation. Qualitative variables were presented in percentages. The probit analysis was used for the determination of IC₅₀. The analysis of variance (ANOVA) has a single factor and *Post-hoc* Turkey and the Krustal-Wallis non-parametric test were used to assess the influence of the extracting solvent on total polyphenols and flavonoids content. The Pearson correlation coefficient used to test the degree of association of the antioxidant potentialities of the extracts and their polyphenolic content. The level of significance is set at 5%.

3. RESULTS

3.1 Extraction Yield

The Fig. 1 shows the extracts obtained from the crude extracts according to the different solvents. Results presented in this figure indicate that extraction yields vary from one plant species to another and from one extract to another. In general, mixed (water-ethanol) and ethanolic solvents have been particularly effective in improving the extraction yield.

3.2 Total Polyphenols and Flavonoids Content

The quantification of total polyphenols and flavonoids in extracts is determined from the

linear regression equations of the calibration curve of gallic acid ($y = 0.0012x - 0.0388$ with $R^2 = 0.9988$) and the rutin equations ($y = 44.135x - 0.1893$ with $R^2 = 0.9909$). This content expressed respectively in mg equivalent of gallic acid /g of extract for the total polyphenols and μg equivalent of Rutin /g of extract for the flavonoid. Fig. 2 shows the results obtained for the quantification of total polyphenols of the crude extracts of the four plants studied. From this figure, there is a variation of total polyphenols content according to plant species and extract type. A comparative analysis of these data shows that the extraction solvents influence the polyphenol content of the various extracts from the plants studied. In general, aqueous extracts from the plants studied showed a low total polyphenols content, unlike ethanolic and hydro-ethanolic extracts. Thus, the total polyphenol content of the hydro-ethanolic extract of each of the plants studied was significantly higher than that of the aqueous and ethanolic extractions ($P < 0.05$). Also, the total polyphenol content did not vary significantly for the aqueous extract and ethanolic extract ($P > 0.05$) of each of the plants studied. This indicates that the mixed solvent (water-ethanol) extracts more polyphenols than pure solvents (water and ethanol).

Fig. 3 presents the total flavonoids content of extracts of the studied plants. Analysis of the data in this figure indicates a variation of the total flavonoid content following the extracts from each plant. In general, aqueous extracts from the plants studied showed a low total flavonoid content, unlike ethanolic and hydro-ethanolic extracts. Thus, compared with the aqueous and ethanolic extracts, a significantly ($P < 0.05$) high total flavonoid content was obtained for the hydro-ethanolic extract of *G. ternifolia* and *G. kola* ($P < 0.05$). In contrast, the ethanolic extract of *C. filiformis* showed a significantly ($P < 0.05$) high flavonoid content compared with the aqueous and ethanolic extracts of this plant. For *R. coccinea*, ethanolic and hydro-ethanolic extracts had a significantly high flavonoid content compared to aqueous extract.

3.3 Antioxidant Activity of Plants

3.3.1 Trapping the free radical of DPPH

All the extracts tested reduced the DPPH radical to varying proportions. This inhibition reflecting the antioxidant activity of the extract is proportional to the increase in the concentration of extracts. The power of the inhibitory capacities of the DPPH radical of the various extracts and

reference molecules expressed in IC_{50} presented in Fig. 4. As IC_{50} is inversely proportional to the antioxidant potential of the extract, more the value of IC_{50} is low, more the antioxidant activity of the sample is better. From this figure, it should be noted that the different extracts have a variable antioxidant power depending on the plant and the type of extract. Overall, all plant extracts from the plants studied have a low antioxidant power relative to vitamin C. The same observation is made for BHT except for the semi-ethanolic extract of *R. coccinea*, which has an antioxidant power similar to that of BHT. A comparative analysis of the antioxidant powers of the extracts of the plants studied indicates that the best antioxidant activities were obtained for the hydro-ethanolic extracts.

3.3.2 Iron reducing the power of extracts (ferric reducing-antioxidant power)

The various extracts from the plants studied showed a reducing potential of the ferri-iron indicated by the increase in optical density proportional to the increase in the concentration of extracts of the various vegetable species. The reduction of the ferri-iron (IC_{50}) of the various extracts and the reference standards were determined and the results are presented by Fig. 5 Comparative analysis of the data in this figure shows that the different extracts have a ferric reductive power that varies depending on the plant and the type of extract. In general, the ethanolic and hydro-ethanolic extractions presented the best ferric iron-reducing activities. For example, the hydro-ethanolic extract was been more active on the reduction of ferric iron for the species of *G. ternifolia* and *C. filiformis*. On the other hand, the ethanolic extract showed a better reductive power of iron for *R. coccinea* and *G. kola*. Moreover, a comparison of the ferric reductive power of extracts with the reference standards shows that all extracts of *C. filiformis* and *G. kola* are more reductive than vitamin C. However, the ferric reducer potential of BHT is better than that of *G. ternifolia*, *R. coccinea* extracts and *G. kola* aqueous extract. All the extracts of *C. filiformis* and hydro-ethanolic and ethanolic extracts of *G. kola* have a ferric reductive activity similar to that of BHT.

3.3.3 Correlation between total polyphenol content and antioxidant activity

In general, the biological and pharmacological activities of plant species are attributed to the secondary metabolites present in these species. Table 2 shows the degree of association

between the total polyphenol content and the antioxidant activity of the four plants studied. Analysis of the data in this table reveals that

there is a strong positive linear correlation between the total polyphenol content and the antioxidant activity of the plants studied.

Table 2. Correlation between total polyphenol content and antioxidant activity

Correlation (Pearson)	<i>G. ternifolia</i>		<i>R. coccinea</i>		<i>G. kola</i>		<i>C. filiformis</i>	
	TPT vs DPPH	TPT vs FRAP	TPT vs DPPH	TPT vs FRAP	TPT vs DPPH	TPT vs FRAP	TPT vs DPPH	TPT vs FRAP
R	0.847	0.819	0.981	0.971	0.985	0.977	0.99	0.923
P value	0.002	0.04	0.0001	0.001	0.0001	0.001	0.0001	0.009
Significance	**	*	**	**	**	**	**	**

* : significant ($p < 0.05$); ** : very significant ($p < 0.01$)
 NS: No significant; TPT: Total Polyphenol content

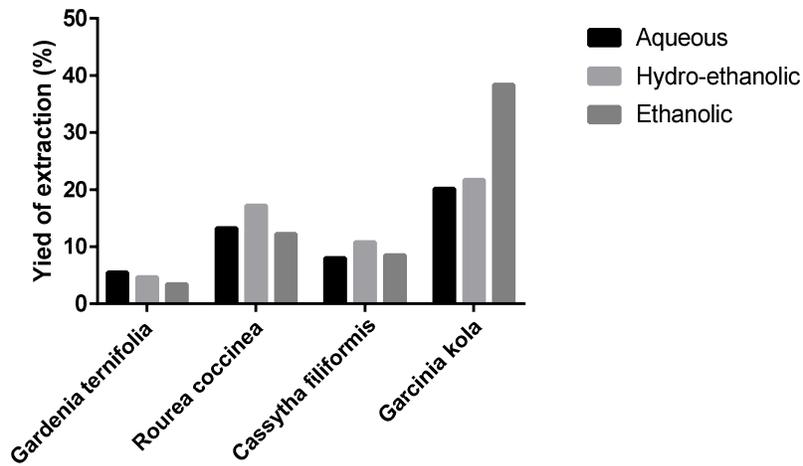


Fig. 1. Yield of crude extracts

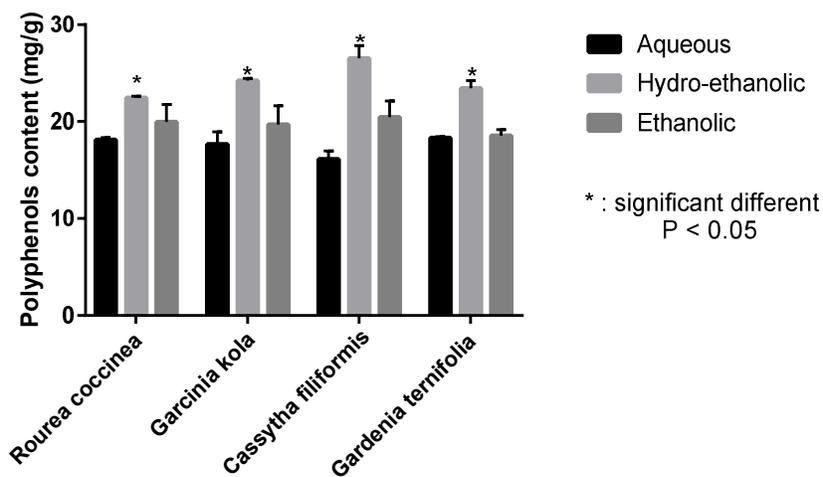


Fig. 2. Total polyphenols content of extracts of studied plants

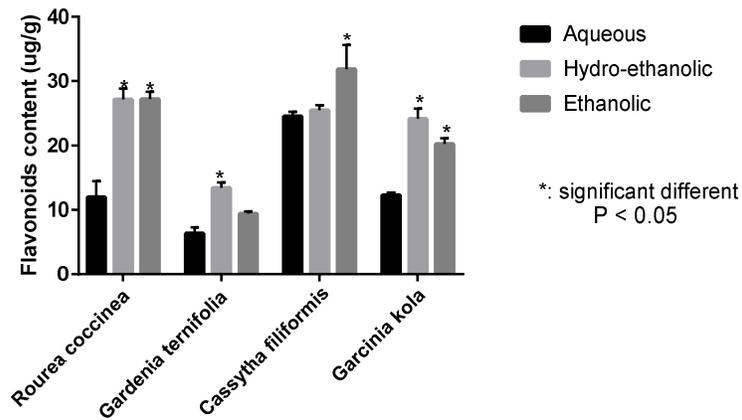


Fig. 3. Total flavonoids content of the various extracts of studied plants

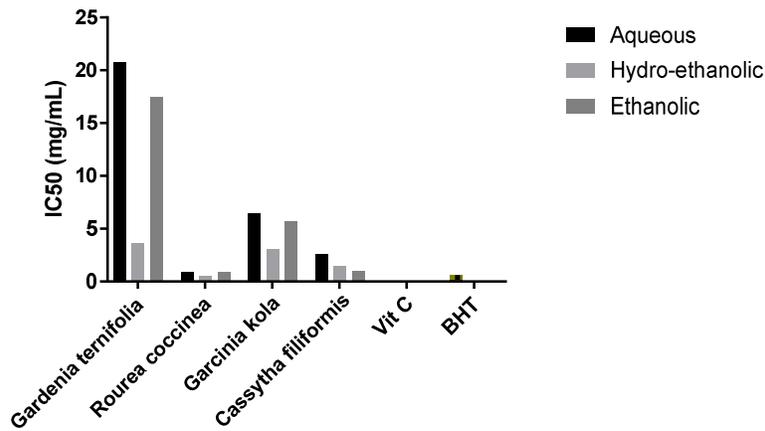


Fig. 4. Anti-radical power (IC₅₀) of plant extracts and reference molecules

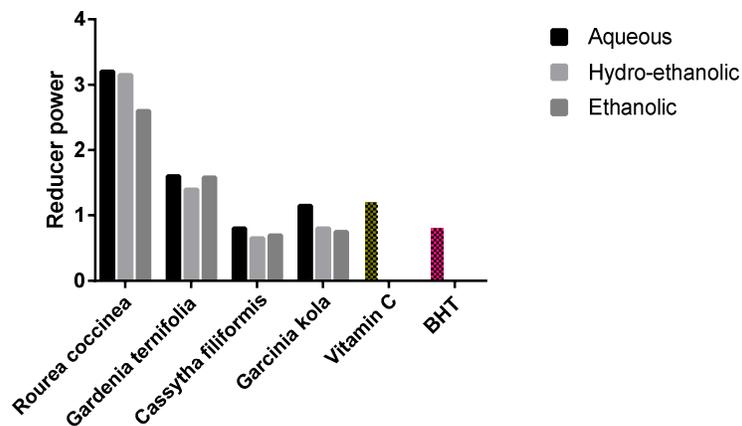


Fig. 5. Reducing power (IC₅₀) of plant extracts and reference

4. DISCUSSION

In this study, the extraction yields obtained varied from one plant species to another and from one extract to another. The best yield was obtained for the hydro-ethanolic and ethanolic extractions. These observations show that the mixed solvent (water-ethanol) offers a better quantitative bioavailability of the active ingredients. In the literature, several studies have reported that the equal volume mixed solvent improves the extraction yield more than pure solvents [19–21]. This extraction capacity of this solvent, therefore, justifies the observed yield and the choice given to water and alcohol as a solvent for preparing medicinal recipes used in traditional Beninese medicine.

The results of this study have shown that extracts from the plants studied have variable content of total polyphenols and flavonoids. This total polyphenol content significantly influenced by the solvents used for extraction. Thus, high levels of total polyphenols were obtained for hydro-ethanolic extracts. These findings support those of several authors who have shown that mixed solvents are very effective in polyphenol extraction [19,20,22,23]. This high extraction power of bioactive compounds from mixed solvents (water-ethanolic) is due to the increase in the solubility of these compounds in this type of solvent [24]. However, Venkatesan et al. [25] have shown that mixed solvents obtained a mixture of organic solvent and water in a ratio of 40% water have a better extraction capacity of phenolic compounds than those made with more than 40% water. Moreover, Labre [26] points out that hydro-ethanolic extracts have high stability, an unlimited shelf life, ease of administration and rapid gastrointestinal absorption. These data may favour the choice of this type of extract for pharmacological tests. Very few scientific studies have examined the quantification of total polyphenols and flavonoids for most extracts from the plants studied. The few data present in the literature concerned *G. kola* and *C. filiformis*. Indeed, in southern Nigeria, Ukaoma et al. [27] indicated a slightly high polyphenol content (23.2 mg/g) and flavonoids (4 mg/g) for the ethanolic extract of the seeds of *G. kola* compared with the data obtained in this study. This trend contrasts with the low level reported by Yété et al. [28] for the dry matter of the seeds of *G. kola* harvested in the Ouémé department in Benin (polyphenols content $1,65 \pm 0.08$ mg/g and flavonoids 0.35 ± 0.30 mg/g). Moreover, Adebayo et al. [29] showed that the methanolic extract of the seeds

of *G. kola* was very low in polyphenols 0.31 ± 0.52 mg/g and flavonoids 0.05 ± 0.01 mg/g of extract. Concerning *C. filiformis*, the study of Sakshy et al. [30] carried out in India showed that the ethanolic extract of the plant *C. filiformis* is less rich in total polyphenols (15 mg/g) of extract than the plant of this study (20.44 mg/g). These observations reflecting a discrepancy between the different results can be explained by several factors such as the phenolic composition of extracts [31], genotypic factors [32], biotic conditions (species, organ and physiological stage) and abiotic conditions [33], the nature of the soil and the type of microclimate and also the bioclimatic stages where these plants grow [34]. Moreover, two of the plants studied, *R. coccinea* and *G. ternifolia*, did not have previous scientific studies on the quantification of bioactive compounds from extracts from plant organs studied. Nevertheless, literature data indicate that *R. coccinea* is a medicinal plant whose leaves and roots are very rich in total polyphenols and flavonoids [35–37]. *G. ternifolia*, meanwhile, is a medicinal plant that is not very scientifically valued. Its roots are rich in polyphenols and flavonoids [38–41]. This study, providing for the first time data on the quantification of total polyphenols and flavonoids present in different extracts of the plants studied, provides additional data on the phytochemical profile of leaves of *R. coccinea* and roots of *G. ternifolia*.

The results obtained for antioxidant tests showed that antioxidant activity varies with plant and extract type. In general, the hydro-ethanolic extracts of the different plants studied were more active in inhibition of the DPPH radical and reduction of ferric iron. This is significantly correlated with the high total polyphenol content of this extract type. Several scientific studies have shown that polyphenols in plant extracts are responsible for their antioxidant activity [1,23,42–44]. In the literature, unlike the plants of *G. ternifolia* and *R. coccinea*, several scientific studies have referred to the *in vitro* antioxidant potential of extracts from plants of *G. kola* [45–47] and *C. filiformis* [30,48,49]. Concerning *R. coccinea*, although none *in vitro* antioxidants activities from the leaves of the plant have been investigated, two scientific studies referring to the antioxidant properties *in vivo* of the leaves of the plant have been identified [50,51]. Moreover, several studies showed that *R. coccinea* roots have *in vitro* and *in vivo* antioxidant potentials [37,52]. These observations indicate that the *R. coccinea* plant has antioxidant potential, which

would probably justify its effectiveness in treating diseases where oxidative stress is involved such as diabetes, hypertension, etc. Like *R. coccinea*, the literature data concerning *G. ternifolia* did not mention the *in vitro* antioxidant potential of extracts of the roots of the plant. However, studies of Awas et al. [39] and Mpiana et al. [53] showed that the leaves of the plant have a powerful antioxidant activity.

5. CONCLUSION

The present study revealed that the plants of *G. ternifolia*, *R. coccinea*, *G. kola* and *C. filiformis* have a polyphenol and flavonoid content that varies according to extracts. These plant species have antioxidant activity that varies with the type of extract. However, of the three extracts produced, the hydro-ethanolic extractions showed the high levels of total polyphenols and flavonoids corroborating their best antioxidant activities. These observations justify the use of these plants in traditional African medicine for pathologies associated with oxidant stress. However, in-depth phytochemical investigations should be carried out on the antioxidant properties *in vivo* of the extracts of the plants studied for better qualification.

CONSENT

As per international standard or university standard was written patient consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard was written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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