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Phytochemical Study and Antioxidant Activities on Extracts of the Leaves and Roots of *Costus afer* Ker Gawl. (Zingiberaceae)

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ABSTRACT

The extracts of leaves and roots of *Costus afer* plant have been fully investigated in an attempt to determine their phytochemical constituents and antioxidant activities. Phytochemical screening carried out using thin layer chromatography (TLC) revealed the presence of several secondary metabolites in all the selected extracts of the plant. In addition, our results showed that the leaves of *C. afer* are the richest in polyphenols with an average value of 3416.25 µg EAG/g MS. The total flavonoid assay revealed a highest content in the leaves (8.02 %). Furthermore, studies of the antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH•) method showed a significant effect compared to the reference vitamin C. Ethyl acetate extract of the leaves show a higher percentage of inhibition (83 %), followed the roots (69 %).

Keywords: Zingiberaceae, *Costus afer*, Antioxidant, Polyphenols, DPPH.

INTRODUCTION

The inceasing number of people affected by Hemorrhoids is a public health concern and the numbers are expected to increase if nothing is done. Approximately 10-25% of the tested population have this disease. This disease is commonly located around and inside the anus and rectum and can go unnoticed because it is painless. Severe situations of hemorrhoid will cause the dilation of veins around the anus[1]. However, given the increasing number of cases and the complications associated with this

disease, it could have long-term socio-economic repercussions on the rural population as current drug treatments remain insufficient. Faced with this health crisis, scientists are continuously searching for ways to mitigate the disease by studying plant extracts. Plant extracts have shown tremendous therapeutic properties because they contain active agents that can act against the disease[2-6]. There is increasing need in the use of plant extracts in the developed world against many diseases. This trend is also observed in many developing countries especially Ivory Coast, where Zingiberaceae plant is currently being used as a traditional medicine against hemorrhoid[7]. In this research, we will present results on the phytochemical studies and antioxidant properties on the leaves and roots extracts of *Costus afer*. This work is done in an attempt to increase on the use of medicinal plants in Ivory Coast, especially *Costus afer* with limited literature.

MATERIAL AND METHODS

Materials

Plant material

The leaves and roots extracts of *C. afer* were collected in July 2017 at Aboisso (South East of Ivory Coast). This was received and identified by herbariums in the Centre National de Floristique at the University of Félix Houphouët Boigny with reference number CNF 17212. The leaves and roots of *C. afer* plant were thoroughly washed with water, air-dried for one month and three days and pulverized by means of an artisanal mill.

Equipment Used

Technical equipment used in this research are: a precision balance (Denver, S-234 series, Max 230 g), a Buchner, a UV lamp (254 and 366 nm), a spectrophotometer (AL8000Aquatic series), a rotary evaporator of HEIDOLPH1 type and an electric dryer. The deposits were carried out on chromatographic plates (silica gel 60 F254, aluminum, 20 × 20 cm, Merck).

Methods

Extraction of secondary metabolites

Hydromethanol maceration

5.00 g of each powder was macerated in 50.00 mL of methanol (80%) with constant stirring for 24 hours. The resulting product was obtained through suction filtration and stored between 4 - 5°C in a refrigerator. This operation was repeated twice, keeping the same pomace, but with a renewal of the solvent. The crude leaves and roots hydromethanolic extracts from *C. afer* were obtained using a rotary evaporator. These extracts were dried in an oven and weighed using a precision balance (Denver, S-234 series, Max 230 g) to determine the yield. These extracts were used to prepare the selective extracts for subsequent assay of polyphenols and flavonoids.

Preparation of selective extracts

Extracts from leaves and roots were respectively treated with the following increasing polarity solvents: hexane, chloroform, ethyl acetate and n-butanol 20.00 mL of the crude hydromethanolic extract was successively soaked with 3 x 10 mL of hexane, chloroform, ethyl acetate and n-butanol. The different fractions obtained from these solvents (hexane, chloroform, ethyl acetate and n-hutanol) were concentrated on a rotary evaporator at 50°C and then stored in a refrigerator (4-5°C) for subsequent use in phytochemical screening and antioxidant activity.

Phytochemical screening of selective extracts

Choice of phytochemical screening method

Phytochemical screening of the selective extracts were carried out using either thin layer chromatography (TLC) or characterization reactions in a liquid medium. In this research, we use the method of chemical screening by TLC according to reports from Békro *et al.,* Mamyrbekova- Bekro *et al.,* and N'gaman *et al.,*[8,9,10,11]. The different developers used for the phytochemical screening by TLC are shown in Table 1.

Table 1: Reagents and revealed compounds

Reagents	Revealed compounds
Liebermann-Bürchard	Sterols and terpenes
Godin	Sterols, terpenes and flavonoids
Test on the lactone ring, KOH	Coumarins
Shinoda's test (Cynanidin reduction test)	Flavonoids
Dragendorff	Alkaloids
Iron chloride ($FeCl_3$)	Tannins and polyphenols

Detection of sterols and terpenes

Liebermann-Büchard's test: In a test tube, an aliquot of the extract was diluted with 1.00 mL of acetic anhydride and 0.50 mL of concentrated H_2SO_4 added slowly on the walls of the test tube. The appearance of a purple color turning blue to

green indicates a positive reaction for the presence of steroids and terpenes.

Detection of flavonoids, coumarins, sterols and terpenes

Godin test: The sample is prepared by mixing equal volumes of a 1% (v/v) ethanol - vanillin solution with 3% (v/v) perchloric acid solution. Chromatoplates were sprayed with this reagent, then with an ethanolic solution of H_2SO_4 at 10% (v/v). The resulting mixture was heated to 100°C until spots of various colors appear. The colours observed were; blue for sterols, triterpenes and coumarins; purple for sterols and polyterpenes; yellow and orangerose for flavonoids and green for triterpenes.

Detection of flavonoids, coumarins

Potassium hydroxide (KOH) test: Prepare methanolic solution of KOH (5%, m/v) by dissolving 5.00 g of KOH in 100.00 mL of methanol. After spraying the chromatographic plate, coumarins appear in the form of yellow spots in the visible and UV regions (336 nm).

Detection of coumarins

Test on the lactone ring: 2.00 mL of aqueous extract were placed into two separate test tubes. In one of the test tubes, was added 0.50 mL of 10% NaOH (w/v), then the test-tubes were heated in a hot-water bath until boiling. After cooling at room temperature, 4.00 mL of water were added to each test tube. If the liquid is transparent or transparently yellowish (yellow) relative to the liquid from the test tube containing no alkaline solution, the reaction is positive. Acidifying the transparent solution with a few drops of concentrated HCl, lead to the lost of the yellow color, forming a cloudy suspension or a precipitate.

Detection of flavonoids

Shinoda's test (Cynanidin reduction test): 5 to 7 drops of concentrated HCl and 10 to 15 mg of Zn or Mg shavings were added to 2 mL of the aqueous extract. After 3 to 5 min a red-orange colour was observed indicating the presence of flavonoids; coloration characterizes the flavonoids. Heating the mixture in a hot water bath for 2 to 3 min can increase the rate of the reaction.

Detection of alkaloids

Dragendorff's test: The extract was dissolved in 6 mL of ethanol and 2 drops of Dragendorff's reagent was added. An orange precipitate or solution indicates the presence of alkaloids.

Detection of polyphenols

Iron(III) chloride ($FeCl_3$) test: To 2 mL of the aqueous extract, we added a few drops of an aqueous solution of $FeCl_3$ 2% (w/v). A\ dark blue or dark greencolor indicate the presence of polyphenols.

Determination of total polyphenols

Total polyphenol content was determined using the colorimetric method proposed by Folin-Ciocalteu and used by Kabran, Kadja, N'guessan and N'Gaman[11,12,13,14]. To a 1 mL solution of each extract diluted to 1/20 with distilled water, we added 1.5 mL of Na_2CO_3 (17%, w/v) and 0.5 mL of Folin-Ciocalteu reagent (0.5 N). The whole mixture was incubated at 37°C for 30 min the absorbance was recorded at 720 nm against a blank without the extract taken as a reference.

In addition, the determination of total polyphenols was done using the linear calibration line (y = ax + b) carried out by a standard extract of gallic acid at different concentrations (0 µg/mL to 1000 µg/mL) under the same conditions as the sample. The results are expressed in microgram gallic acid equivalent per gram of the dry matter (µg EAG/g DM) of the powdered plant.

The total polyphenol content (Q) was calculated using the formula[17]:

$$Q = \frac{v \times c \times d}{m}$$

Where; v = final volume of the extract (mL); c = concentration of the extract (µg/mL), d = dilution; m= mass of dry matter of the hydrolyzed plant material (g), Q = (µg EAG/g MS).

Determination of total flavonoids please use this F(%) below

Total flavonoid assay was performed using the method proposed by Hariri *et al.,*[15] and used by Kabran[12]. In this method, 2.00 mL of each hydro-methanolic extract was diluted to 1/ 20 with distilled water and mixed with 100 µL of Neu reagent. The absorbance was read at 404 nm and the result compared to that of quercetol taken as standard (0.05 mg/mL), diluted under the same conditions and treated with the same amount of reagent. The percentage of total flavonoids was calculated in quercetol equivalent according to the following formula below proposed by[11,12,17]:

$$F\,(\%) = \frac{\left(\frac{0,05 \times Aext}{Aq} \times 100 \times d\right)}{Cext}$$

Where; F(%), = percentage of total flavonoids; Aext = Absorbance of the extract; Aq = Absorbance of quercetol; d = dilution; Cext = Concentration of extract (mg/mL)

2,2-diphényl-1-picrylhydrazyl (DPPH) radical scavenging activity

The spectrophotometric evaluation of the antioxidant activity of the selective extracts were done using the method proposed by Popovici *et al.,*[16] and repeated by Bea *et al.,*[17]. 2,2-diphényl-1-picrylhydrazyl (DPPH•) was dissolved in absolute ethanol to obtain a concentration of 0.02 mg/mL. Similarly, solutions of the extract were prepared in absolute ethanol with varying concentrations (0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL and 3.0 mg/mL). In a dry and sterilized test tube, 0.5 mL of the different extract concentrations were mixed with 1.5 mL of the solution of DPPH at 30 min interval. The resulting mixture was thoroughly mixed and placed in the dark for 15 minutes. The absorbance of the mixture was measured using a spectrophotometer to be 517 at the maximum wavelength of DPPH• check an confirm the absorbance value please. The absorbance was measured at 3 min interval for 15 min and this process was repeated for the different extract solutions. Ascorbic acid (vitamin C) was used as the reference positive control. Scavenging effect was calculated as[17]:

$$\% I = (A_0 - A_1) \times 100/A_0$$

Where A_0 = absorbance of the control; A_1 = absorbance of the extract.

A standard graph of the extract concentration versus the scavenging activity was plotted and the concentration efficiency of the plant extract at 50% reduction of 2,2-diphényl-1-picrylhydrazyl (DPPH) (IC_{50}) was determined by extrapolation[12,17]

RESULTS AND DISCUSSION

Phytochemical Screening

Extracts with Hexane

For the detection of secondary metabolites, we used the solvent system: Hexane/AcOEt (8: 2; v/v). Before spraying, we observed three spots for the leaf extract and one spot for the root extract. In the UV region at 254 nm, we observed three spots for the leaf extract and five spots for the root extract. In addition, at 365 nm, we observed ten spots for the leaf extract and four spots for the root extract.

In this method, family-specific developers were used to determine large families of secondary metabolites.

With the Liberman-Bürchard reagent, we observed nine spots for the leaf extract and eight spots for the root extract. With Godin's reagent, we identified six spots for the leaf extract and twelve spots for the root extract. The use of sulfuric Vanillin solvent gave eleven spots for the leaf extract and twelve spots for the root extract.

Potassium hydroxide (KOH) specific to coumarins, gave us four spots for the leaf extract and three spots for the root extract at 365 nm in the UV region. In short, we would note that the plant organs would contain sterols, flavonoids and coumarins (blue or light blue, purple or Brown, purple or light purple, yellow, red, orange, gray spots). Thus, by comparing the R_f of the spots observed, the leaf extracts with hexane would contain six spots which have been identified as sterols, two spots identified as terpenes; four spots identified as coumarins and five spots identified as flavonoids. In addition, with the root extract, we had two spots identified as sterols; four spots identified as terpenes; three spots identified as coumarins and seven spots identified as flavonoids. Previous reports isolated these metabolites from the leaf extract with hexane in *Costus afer*[18].

Table 2: Screening of the leaf and root extracts of *C. afer* with hexane

Secondary metabolites	Leaves	Roots
Sterols and terpenes	++	++
Coumarins	++	++
Flavonoids	++	++

(++) = abundance

Extracts with Chloroform

The solvent system: $CHCl_3$/AcOEt/Hexane (5:6: 2.5 ; v/v/v) was used to determine the secondary metabolites.

Before spraying with the solvent system, no spot were visible.

In the UV region at 254 nm, the chromatograms presented four gray spots for the leaf and then three gray spots for the root extracts. In addition, four spots were observed for the leaf and two spots for the root extracts in the UV region at longer wavelength (365 nm). After treatment with Neu's reagent, we observed six spots for the leaves and four spots for the roots. Further treatment of the extracts with $AlCl_3$ gave six spots for the leaves and six spots for the roots.

These results would show that our compounds are indeed flavonoids (blue or light blue, purple or light purple, yellow, green, orange spots). Thus, by comparing the R_f values of the spots, the chloroform extract would contain five spots, which can be identified as flavonoids (three spots for the leaves "$R_f = 0.17$; $R_f = 0.24$; $R_f = 0.31$" and two spots for the roots "$R_f = 0.15$; $R_f = 0.24$").

Table 3: Screening of extracts with chloroform on the leaves and roots of *C. afer*

Secondary metabolites	Leaves	roots
Flavonoids	++	++

(++) = Abundance of compound

Extracts with Ethyl acetate

In the method of identification of secondary metabolites in ethyl acetate extracts, the solvent system $CHCl_3$/AcOEt/CH_3CHOOH (6.5:3:0.5 ; v/v/v) has been used. In the visible region, observed two yellow spots for the leaves and three yellow spots for the roots.

In the UV region at 254 nm, we observed five spots for the leaves and twelve spots for the roots for the ethyl acetate extract. In addition, four spots were observed for the leaves and seven spots for the roots in the UV region at a higher wavelength of 365 nm.

After spraying, the chromatoplates showed various stains with the Neu reagent. We observed seven spots for the leaves and eight spots for the roots. With $AlCl_3$ at 365 nm in the UV region, we observed three spots for the leaves and seven spots for the roots. With $FeCl_3$, five spots were observed for the leaves and six spots for the roots.

These results would reflect the actual presence of flavonoids and tannins (blue or light blue, purple or light purple, yellow, green, black or gray spots). Thus, by comparing the R_f of the spots, the ethyl acetate extract would contain a total of nine spots which have been identified as flavonoids: five in the leaves of $R_f = 0.04$; $R_f = 0.10$; $R_f = 0.19$; $R_f = 0.36$ and $R_f = 0.56$ and four in the roots of $R_f = 0.15$; $R_f = 0.36$; $R_f = 0.51$; $R_f = 0.77$. Then four spots identified as tannins: one in the leaves of $R_f = 0.07$ and three in the roots of $R_f = 0.20$; $R_f = 0.43$ and $R_f = 0.58$. These metabolites have been isolated from ethyl acetate extracts from the leaves and roots of a variety of *C. afer* from Cameroon by other authors[18].

Table 4: Screening of extracts with ethyl acetate on the leaves and roots of *C. afer*

Secondary metabolites	Leaves	roots
Flavonoids	++	++
Tannins	+	++

(+) = presence, (++) = Abundance of compound

Extracts with n-butanol

In the phytochemical screening of the extracts with n-butanolic, we used the AcOEt/MeOH/H_2O/CH_3CHOOH system (6:0.75:0.7:0.25; v/v/v/v) as eluent.

Before spraying, four spots and one spot on extracts of the leaves and roots respectively.

In the UV region at 254 nm, five spots were seen on extracts of the leaves and seven spots extracts of the roots. In addition, at 365 nm we observed seven spots on the extracts of the leaves and six spots on extracts on the roots.

After spraying with Neu's reagent, we observed nine spots on extracts of the leaves and ten spots on extracts of the roots. Furthermore, when $AlCl_3$ reagent was used at 365 nm in the UV region, five and seven spots were respectively observed on the extracts of the leaves and roots. Further analysis with $FeCl_3$ reagent gave tow brown spots each on the extracts of the leaves and roots.

Using the potassium hydroxide reagent (KOH) at 365 nm, we were able to observe five spots on extracts of the leaves and seven spots on extracts of the roots. Using specific developers of the alkaloids together with Dragendorff reagent gave seven spots on extracts of the leaves and no spots on extracts of the roots.

The results on the extracts reveal the presence of flavonoids, tannins, coumarins and alkaloids (blue, fluorescent blue, yellow, black, Orange, red and gray spots). Comparing the retention factors, Rfs on the spots, extracts with n-butanol had twelve spots in total, which were identified as flavonoids seven from extracts of the leaves ($R_f = 0.11$; $R_f = 0.16$; $R_f = 0.24$; $R_f = 0.33$; R_f

= 0.77; R_f = 0.83; R_f = 0.91) and five from extracts of the roots (R_f = 0.08; R_f = 0.13; R_f = 0.16; R_f = 0.71; R_f = 0.77). In addition, four spots were identified as tannins: two spots from the extracts of the leaves (R_f = 0.69 and R_f = 0.91) and two spots from extracts of the roots (R_f = 0.65 and R_f = 0.75). Six spots were identified as belonging to coumarins: four spots from extracts of the leaves with R_f = 0.08; R_f = 0.12; R_f = 0.20; R_f = 0.77 and two spots from extracts of the roots with R_f = 0.06; and R_f = 0.77. The seven spots identified as alkaloids were all from extracts of the leaves and none for the roots. These results also confirm previous report by Godwill *et al.,* on *C. afer* from Nigeria using extracts with n-butanol[20].

Table 5: Screening of extracts with n-butanol on the leaves and roots of *C. afer*

Secondary metabolites	Leaves	roots
Coumarins	++	++
Flavonoids	++	++
Tannins and polyphenols	++	++
alkaloids	++	-

(+) = Presence; (++) = Abundance of compound, (-) = Absence

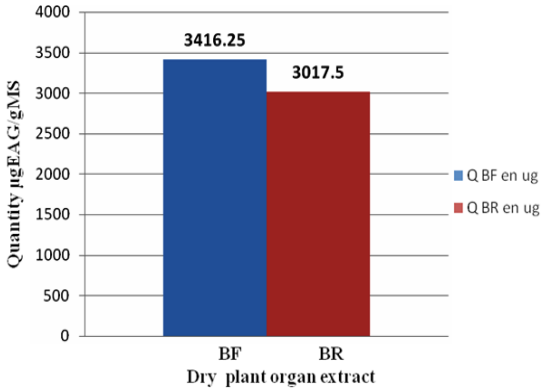
Total polyphenol content

The total phenolic compounds in extracts of the leaves and roots of *C. afer* extracted with hydromethanolic are presented in Fig. 1. From the results, it is observed that the extract from dry leaves gave higher total polyphenol content (3416.25 µg EAG/g MS) with abundance in flavonoids, coumarins and tannins. In addition, the total polyphenol content in extracts of the roots is 3017.50 µg EAG/g MS.

If we compare our results with those obtained from other plants with higher phenolic compounds (5660 µg EAG/g)[21], grape seeds (7500 µg EAG/g)[22], parsley (2802 µg EAG/g), Brussels sprouts (2571 µg EAG/g), lychee (2223 µg EAG/g), broccoli (989 µg EAG/g) and celery (847 µg EAG/g)[23], we can conclude that our extracts are also rich in phenolic compounds.

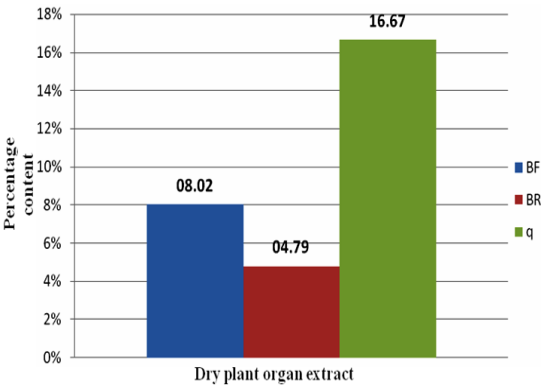
Total flavonoid content

The total flavonoids content in *C. afer* are presented in Fig. 2. From the results, extracts of the leaves have higher flavonoids (08.02%) compared to extracts of the roots (04.79%). Previous reports on the phytochemical screening by TLC also confirm this quantitative experiment of flavonoids in the leaves. The high therapeutic properties of extracts of the leaves are probably due to the high flavonoids content[18].



BF: Leaves and BR: Roots Q : Quercetol

Fig. 1. Total Phenols content of different parts of *C. afer*



BF: Leaves, BR: Roots and q: quercetol

Fig. 2. Total flavonoids content on different extracts of *C. afer*

Antioxidant Activity of *C. afer* using spectrophotometry

2,2-diphényl-1-picrylhydrazyl (DPPH) radical scavenging activity from extracts with n-butanol

The inhibition percentage of 2,2-diphényl-1-picrylhydrazyl (DPPH) radical using extracts with ethyl acetate were done at different concentrations (0.025; 0.05; 1.00; 1.50; 2.00; 3 mg/mL). The results are presented in Figure 3. It is observed that extracts from the leaves have a higher inhibition (3 - 83 %) compared to extracts from the roots (5 - 69 %). Our results however, have lower inhibition percentages compared to the reference, vitamin C.

Determination of inhibition concentrations (IC_{50})

The 50% growth inhibitory concentration of cells (IC_{50}) was determined graphically from regression curves from the percentage inhibition of 2,2-diphényl-1-picrylhydrazyl (DPPH) as a function of the concentration of the leaf and root extracts. The

results are presented in Table 6. From the table, extracts from the leaves have a lower IC_{50} compared to the roots. The IC_{50} of the extracts from the leaves and roots of *C. afer* using ethyl acetate are higher compared to vitamin C. Similarly, the IC_{50} is less than 5 mg/mL[19].

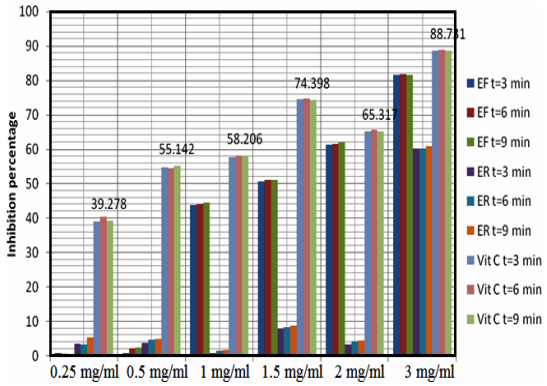


Fig. 3. 2,2-diphényl-1-picrylhydrazyl (DPPH) radical scavenging activity using extracts with ethyl acetate extracts (Leaves and roots)

Table 6: IC_{50} values for the extracts with ethyl acetate and Vitamin C

	t = 3min	t = 6min	t = 9min
ethyl acetate Leaves	1.16	1.17	1.15
ethyl acetate roots	2.92	2.88	2.93
Vitamin C	0.58	0.59	0.61

CONCLUSION

The present study is, therefore, an essential contribution to the chemistry of *C. afer*, in understand the effects and the use of this plant in the treatment of certain diseases in the Ivory Coast. We have successfully presented results on the phytochemical, chromatographic, and antioxidant activities on the medicinal plant *C. afer*. Extracts of the leaves and roots of this plant obtained with different solvents and/or solvent systems revealed the presence of secondary metabolites and antioxidant activities. From the results, hexane, chloroform, ethyl acetate and n-butanol extracts of the plant leaves and roots contain flavonoids, coumarins, tannins, sterols, polyphenols, and terpenes. In addition, alkaloids were present only in the leaves and not in the roots. Our results show higher amounts of polyphenols and flavonoids in the leaves compared to the roots. The total flavonoid assay revealed a high content in the leaves (08.02 %) and high contents of phenolic compounds in the leaves (3416.25 µg EAG/g MS). Furthermore, studies of the antioxidant activities using DPPH• (2,2-diphenyl-1-picrylhydrazyl) method showed a significant effect compared to the standard, vitamin C (88%). The percentages of inhibition were respectively 83 % and 69 % for ethyl acetate extractsof the leaves and roots. The antioxidant activity obtained using spectrophotometric techniquewith 2,2-diphényl-1-picrylhydrazyl (DPPH) radical indicate that both extracts of the leavesand roots have good antioxidant activity, especially with those containing high content of phenolic compounds.

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Conflicts of Interest

The authors declare no conflict of interest.

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