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Phytochemical Screening and Antioxidant Activity of Algerian *Aristolochia longa* Flavonoids

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Abstract

Introduction: *Aristolochia longa*, is widely used as medicinal plant, in the Algerian folk medicine since ancient times and passed through the generations. This study was focuses on the qualitative examination of different phytochemical constituents to determine their antioxidant activities.

Materials and Methods: Leaves and roots were extracted by liquid/liquid extraction technique using the methanol as solvent. The total phenolic and flavonoids content were determined by Folin–Ciocalteu and $AlCl_3$ methods respectively. The antioxidant activity was evaluated with two distinct methods: DPPH radical scavenging assay and ferric reducing antioxidant power test (FRAP). Then, the high performance liquid chromatography (HPLC) method was performed to analyse leaves and roots n-butanol fractions.

Results: Leaves gave significant value of polyphenols (8.580 ± 0.04 mg GAE/g DW). Whereas, n-butanol fraction flavonoids extracted from leaves was observed widely highest in Leaves of Aristolochia longa (4.54±1.94 mg CE/g DW). N-butanol fraction leaves showed a powerful scavenging activity and reducing activity with an IC_{50}=0.044±0.001 mg/mL and EC_{50}=0.126±0.041 mg/mL respectively. The HPLC analysis of leaves and roots n-butanol fraction revealed different bioactive compounds which they belong to the flavonoids category.

Conclusions: The results obtained from this study suggest that *A. longa* leaves were considered as an important resource of flavonoids, which have an interesting antioxidant power.

Keywords: *Aristolochia longa*, Flavonoids, Antioxidant Activity, HPLC, Algerian West Region

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Introduction

The *Aristolochia* species belongs to the *Aristolochiaceae* family, which has up to 500 species.[1] They exhibit an interesting biological properties, e.g. antibacterial, oncological, anti-allergic, a anti-inflammatory, anti-plasmodial, muscle relaxant carcinogen inactivation, anti-proliferation, and stimulation of apoptosis mechanisms.[2-5] The *Aristolochia* genus contain aristolochic acids with is characterized by chronic renal failure.[6] On the other hand, various studies have shown that they have large amounts of secondary metabolites.[7] The *Aristolochia longa* belongs to the genus *Aristolochia* and it is famed as "berrostom" in the Algerian population. Flavonoids present a large group of polyphenols, which normally present in plant tissues with a proportionately concentrations. Their contents in plants depends on many factors such as cultivar, weather, geographical localization, daylight, soil conditions, temperature, water stress, harvesting time.[8] There is a group of about 400 natural polyphenolic compounds located widely in plant.[9] Flavonoids are classified into many subgroups containing the following: flavone, flavanone, flavonol, isoflavonoid, anthocyanin, and chalcones.[9] They are considered as naturals antioxidant drugs compounds, and they also are able to scavenge free radicals, reactive oxygen species and inhibit lipid peroxidation.[9] In addition, their antioxidant activity depends strongly on the number and position of hydrogen of the molecule.[9] In this study deals with, the determination secondary metabolites of *A. longa*. Then, the antioxidant activity of flavonoids fractions was estimated in order to find new bioactive natural products in different parts which were leaves and roots.

Materials and Methods

Collection and Preparation of Plant Material

Aristolochia longa leaves and roots were collected in Mach

2015 in Ouled Ali region located in Tissemsilt city (Algeria). The plant was deposited in the Nature and Life Science Faculty, University of Mascara, Algeria, as a voucher specimen (ARI0001). The leaves and roots were dried, reduced to powder and then stored in closed container away from heat, air and light until moment of use.

Phytochemical Screening

Flavonoids, alkaloids, glycosides, steroids, saponins, coumarins, terpenoids and anthraquinones were subjected to a phytochemical screening using standard procedures according to the methods described by.[10-12]

Extraction of Flavonoids

Ten grams of plant powder (leaves and roots) was macerated in 100 mL of pure methanol for 24 hours for three times. The mixture was filtrated through Whatman n°2. Then, the methanol was removed using rotary evaporator at 60°C. The obtained dry residue was treated with 50 mL of warm water for the purpose of obtaining an aqueous extract. The aqueous extract was subjected to a series of liquid-liquid extraction. This operation allows the separation of one or more constituents by the use of their unequal distribution in two substantially immiscible liquids. It consists in the addition of 3 × 30 mL of chloroform, which eliminates chlorophyll (leaves) and lipids (roots). Then diethyl ether was added to extract the free aglycones and flavonoids. Finally, the addition ethyl acetate allows to eliminate the monosids and the majority of flavone glycosides. After each phase, the aqueous phase was recovered. At the end, n-butanol was added to recover the alcoholic phase. All the obtained fractions were evaporated and dried.[13]

Extraction Yield

The extraction yield was calculated according to the formula:

$R\% = Pe/Pp \times 100$

R: Yield

Pe: weight of the extract

Pp: weight of the plant

Determination of Total Phenolic Content

The total phenolic content of the extracts was determined by using the method of Folin-Ciocalteu.[14,15] A sample of 200 µL of the extract was mixed with 1 mL of Folin-Ciocalteu reagent (10%) and 0.8 mL of 7.5% sodium carbonate (Na_2CO_3). The mixture was incubated at room temperature for 30 minutes. Then, the absorbance was determined at 765 nm using a spectrophotometer (UV mini-1240-vis -SHIMADZU). The calibration curve was prepared using gallic acid as standard. The results are expressed in milligrams equivalent of gallic acid per gram of dry extract (± SD for tree replications).

Determination of Flavonoids Content

The quantitative determination of flavonoids was calculated by spectrophotometric assay according to the method described by.[16] A sample of 500 µL of the extract was combined with 1500 µL of distilled water. Then, 150 µL of a 5% $NaNO_2$ solution was added. After 5 minutes, 150 µL of $AlCl_3$ (10%) was added. The solution was homogenized and 500 µL of NaOH was added. The absorbance was determined at 510 nm, using a UV mini-1240-vis -SHIMADZU. The flavonoids content was expressed in milligrams catechin equivalents (CE)/g of dry extract.

DPPH Radical Scavenging Assay

The antioxidant capacity was determined with DPPH method.[16] A volume of 1.95 mL of DPPH methanolic solution (0.025 g/mL) was added to 50µL of the extracts with different concentrations (from 0.0125 to 5 mg/mL). After 30 minutes in darkness, the absorbance was determined against a blank (extract was replaced with methanol) at 515 nm. Ascorbic acid was used as a standard. IC50 values were determined graphically by linear curve.

Ferric Reducing Antioxidant Power (FRAP)

This test was conducted according to the method of [17] 2.5 mL of phosphate buffer solution 0.2 M (pH 6.6) and 2.5 mL of a potassium ferricyanide $K_3Fe(CN)_6$ at 1% were added to 500 µL of the extract with different concentrations (0.007 to 2.5 mg/mL). After 20 minutes and at 50°C, the composite was incubated in a water bath. A volume of 2.5 mL of trichloroacetic acid (10%) was added. for 10 minutes, the mixture was centrifuged at 3000 rpm. The supernatant (2.5 mL) was combined with distilled water (2.5 mL) and 500 µL of ferric chloride (0.1%). The absorbance was measured at 700 nm.

Identification of Leaves and Roots Polyphenols and Flavonoids byHPLC-DAD-ESI-MS

The identification of *A. longa* leaves and roots polyphenols and flavonoids was conducted on a Shimadzu-system (prominence I. LC-2030C 3D) equipped with a surveyor UV-VIS diode array detection (DAD) and a LCQ advantage max ion trap mass spectrometer (all from Thermo fisher scientific, Waltham, MA, USA), coupled through an electrospray ionization (ESI) source. The separation was performed on ascentis express C18 column (15 cm x 4.6 mm) ID packed with 2.7 µm partially porous particles (Supelco, Bellefonte, PA, USA). The binary mobile phase consisted of water/acetic acid: 0.075% (solvent A) and methanol/acetic acid: 0.075% (solvent B). The gradient was 0-5 minutes: 2% of B, 5-80 minutes: 2%-100% of B, 80-85 minutes: 100%. The flow rate was 0.8 mL/min and the injection volume was 5 µL.

PDA wavelength range was 190–400 nm and the chromatograms were extracted at 280 nm (time constant: 0.025 s; sample frequency: 40 Hz). MS acquisition was performed using an ESI interface, in the negative ion mode, under the following conditions: mass spectral range 100–800 m/z; interval: 0.5 s; scan speed: 1500 amu/s; nebulizing gas (N2) flow: 1.5 L/min; interface temperature: 350°C, heat block: 300°C; desolvation line temperature: 300°C; DL voltage: −34 V; interface voltage: −4.5 kV; Q array DC voltage: 1.0 V; Q array radio frequency voltage: 60 V.

The quantitative of each compound was carried out by the external standard method in a concentration range of 1–100

ppm. The results were obtained from the average of three determinations and are expressed as microgram per gram dried extract ± %RSD.

Statistical Analysis

All antioxidant activities data of several parts were done in replicate. The results were expressed as mean values ±standard deviation (SD). The means were compared by using the one-way analysis of variance (ANOVA). The differences between individual means were deemed to be significant at $P < 0.05$.

Results

Phytochemical Screening

Flavonoids, tannins, coumarins, saponins, alkaloids and steroids were found in leaves and roots of *A. longa* powder. The result was presented in Table 1.

Extraction Yields

As shown in (Figure 1). Methanolic extract leaves yield was found equal to (12.58%) which is highest than the roots yield with a percentage of 9.05%. In addition, the n-butanol leaves gave the highest yield compared to the other fractions (6.5%).

Total Phenolic Content, Flavonoids Content of *Aristolochia longa*

The evaluation of total phenolic compounds was determined by using the Folin-Ciocalteu reagent, which forms blue complexes in the presence of reducing agent.[18] The total phenolic contents in the different extracts were expressed with gallic acid equivalents using the standard curve equation $y=1.216x+0.0054$, $r^2=0.988$. The results showing in Figure 2A revealed that the leaves and roots of *A. longa* contain 8.58 mg GAE/g DW and 8.18 GAE/g DW of total phenolic respectively.

The flavonoids are known by their action to make the yellow or red/blue pigmentations in flowers and provide protection against microorganisms and insects attack.[19] The flavonoids contents (mg/mL) in different fractions of *A. longa* were determined using the standard curve for catechin with the equation $y= 1.8x+0.211$, $r^2= 0.983$.[20] The flavonoids content is highest in leaves methanolic extracts (8.011 mg CE/g DW) than the roots (5.423 mg CE/g DW) (Figure 2B). Table 2 shows the flavonoids contents in various fractions. N-butanol fraction of leaves and roots have the highest flavonoids content (4.54 ± 1.94 and 1.37 ± 0.42 mg CE/g DW, respectively).

Antioxidant Activity Essay

In this study, the antioxidant activity was accessed with two assays, DPPH scavenging activity and FRAP. Scavenging of DPPH free radical determines the potential of the test sample, which shows its effectiveness, prevention, interception and repair mechanism against injury in a biological system.[21] DPPH essay allows estimation of hydrogen radical donating ability of the extract.[22] The blue color DPPH radical present a strong absorbance at 517 nm, which reduce as the color

Table 1. Phytochemical Constituents of the *Aristolochia longa*

Secondary Metabolites	Leaves	Roots
Coumarins	+++	+++
Terpenoids	-	-
Alkaloids	-	-
Saponins	+++	+
Steroids	+++	-
Tannins	+	++
Flavonoids	+++	+

+: Presence; ++Strongly presence; - : Absence

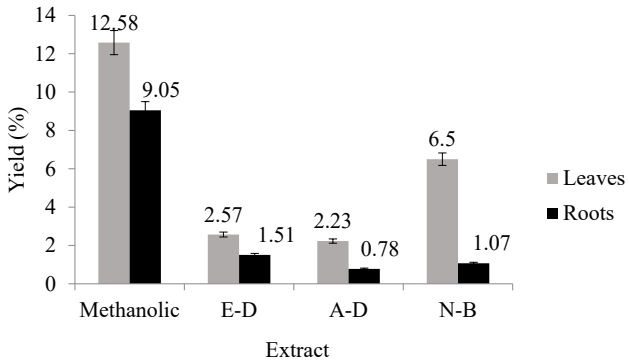

Figure 1. Yield of *Aristolochia longa*. **E-D:** Diethyl ether fraction**; A-D:** Ethyl acetate fraction; **N-B:** N-butanol fraction. Values are expressed as the mean value standard deviation (n=3).

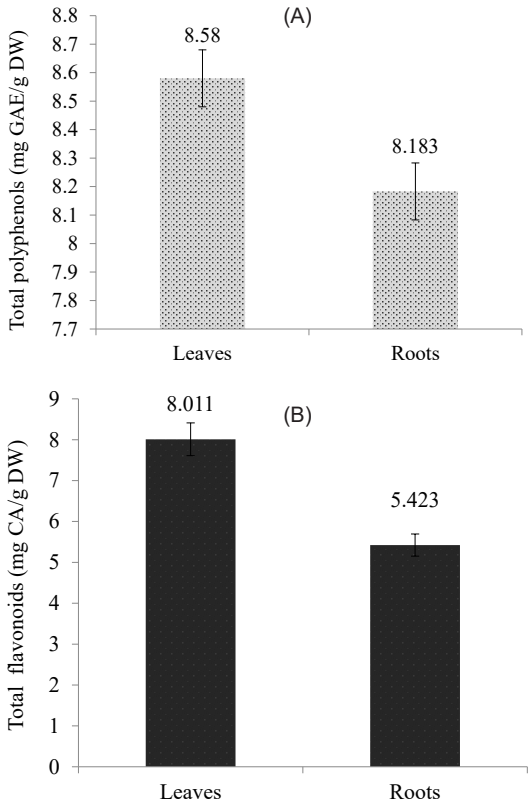

Figure 2. Total polyphenols content (A) expressed on (mg GAE/g DW) and total flavonoids content (B) of methanolic extract of *Aristolochia longa* expressed on (mg CE/g DW). Values are expressed as the mean value standard deviation (n=3).

Table 2. Fractions flavonoids content of *Aristolochia longa* expressed on (mg CE/g DW)

	Flavonoids (mg CE/g DW)		
	E.D	A-D	N-B
Leaves	1.18±0.02	3.05±0.22	4.54±1.94
Roots	0.85±1.47	1.45±1.31	2.87±0.42

E-D: Diethyl ether fraction; A-D: Ethyl acetate fraction; N-B : n-butanol fraction. The values are expressed are means ± SD.

changes.[23] The free radical-scavenging activity was expressed as the IC_{50} values (Table 3). The IC_{50} values of the antioxidant capacity varied significantly ($P<0.05$) from 3.82 ± 0.93 mg/mL to 0.096 ± 0.007 mg/mL in comparison with Ascorbic acid (0.02 mg/mL). However the antioxidant activity (IC_{50}=0.044 ± 0.001 mg/mL) of leaves n-butanol fraction was found non-significant ($P>0.05$). On the other hand, the antioxidant ability (0.096 ± 0.007 mg/mL) of root n-butanol fraction was found significant ($P<0.05$).

The FRAP assay of extracts fractions was determined by the ferric reducing ability. The presence of reducers (antioxidants) causes the transformation of the F^{3+}/ferricyanide complex used in this method to the ferrous form and formation of blue complex, also the reducing power can be determined by measuring the complex at 700 nm. In addition a higher absorbance indicates a higher reducing power.[24] The reducing power of *A. longa* increased with the increase of different fractions concentration and varied significantly ($P<0.05$) from (0.40 ± 0.085 mg/mL to 3.00 ± 0.10 mg/mL) (Table 3). The results of this research revealed that the reducing power of leaves n-butanol fraction (EC_{50}=0.126±0.041 mg/mL) was no significant ($P>0.05$) in comparison with ascorbic acid (EC_{50} = 0.051 ± 0.009 mg/mL).

Identification of Polyphenols and Flavonoids by HPLC-DAD-ESI-MS

The phenolic composition of *A. longa* leaves and root was accomplished by HPLC-DAD-ESI-MS. The chromatograms are presented in Figure 3 and Figure 4. A total of 12 compounds were identified in the n-butanol leaves fraction, 4 phenolic acids (quinin acid, protocatechuic acid hexoside, 3,5-Di-*O*-caffeoylquinic acid and 3,5-Di-*O*-caffeeoylquinic acid) and eight flavonoids (caffeoylhexoside, kaempferol-3-O-rutinoside, naringenin–O-glucoside, apigenin-8-C-hexoside, kaempferol-3-O-glucoside, apigenin-7-O-glucoside, isorhamnetin-3-O-diglucoside and apigenin 6,8-di-C-glucoside). Apigenin-8-C-hexoside was the major identified compounds (57.73%) followed by kaempferol-3-O-glucoside (26.67%), protocatechuic acid hexoside (3.73 %) and the 3, 5-Di-O-caffeoylquinic acid 3.60%) (Table 4). The phenolic profile of the n-butanol fraction of roots extract was less important than the leaves, only nine compounds were identified. The protocatechuic acid hexoside was the major polyphenol in the *A. longa* roots (37.49 %) followed by 3, 5-Di-O-caffeoylquinic acid (15.65%), kaempferol-3-O-glucoside (15.04%) and apigenin-7-O-glucoside (9.87%). The following flavonoids: kaempferol-3-O-rutinoside, isorhamnetin-3-O-diglucoside, and Apigenin 6, 8-Di-C-glucoside were absent in the roots (Table 4).

Table 3. DPPH Scavenging Activity (IC_{50} Values) and Reducing Power (EC_{50} Values) of *Aristolochia longa* Leaves and Roots Fractions

Fractions	Scavenging Activity IC_{50} (mg/mL)	Ferric Reducing Power Activity EC_{50} (mg/mL)
Leaves diethyl ether fraction	1.59±0.09***	1.42±0.03***
Leaves ethyle acetate fraction	0.137±0.001***	0.40±0.085**
Leaves n-butanol fraction	0.044±0.001^{ns}	0.126±0.041^{ns}
Roots diethyl ether fraction	3.82±0.93***	3.00±0.10***
Roots ethyle acetate fraction	2.04±0.5***	1.769±0.4**
Roots n-butanol fraction	0.096±0.007**	0.54±0.24*
Ascorbic acid	0.002±0.0002	0.051±0.009

IC_{50} values are expressed are means ± SD and the IC_{50}, EC_{50} of the deference fractions were provided in terms of mg/mL. Comparison was realized against ascorbic acid, ns: non significance,*$P<0.05$ significant difference, **$P<0.01$very significant difference, ***$P<0.001$ extremely significant difference.

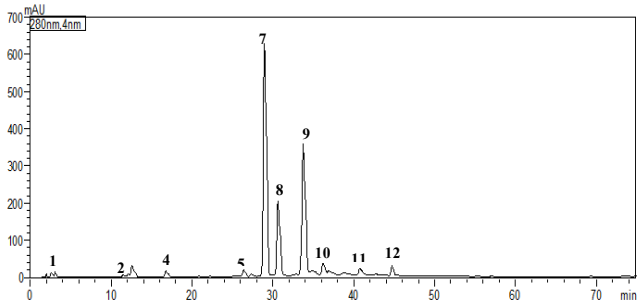

Figure 3. Identification of Polyphenols and Flavonoids From the n-Butanol Fraction of *Aristolochia longa* Leaves by HPLC-DAD-ESI-MS. **1:** Caffeoyl hexoside, **2:** Quinin acid, **3:** Protocatechuic acid hexoside, **4:** 3,5-Di-O-caffeoylquinic acid, **5:** Kaempferol-3-Orutinoside, **6:** Naringenin–O-glucoside, **7:** Apigenin-8-C-hexoside, **8:** Kaempferol-3-O-glucoside, **9:** Apigenin-7-O-glucoside, **10:** 3,5-Di-*O*-caffeeoylquinic acid, **11:** Isorhamnetin-3-*O*-diglucoside, **12:** Apigenin 6,8-di-C-glucoside

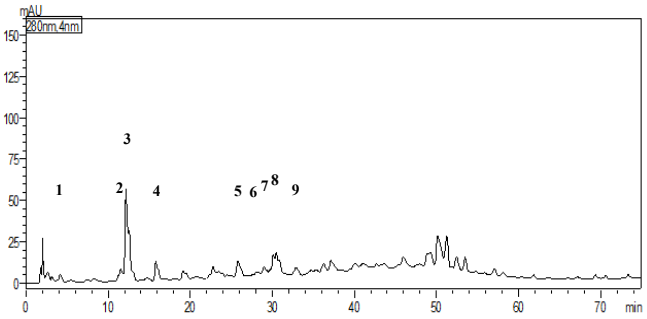

Figure 4. Identification of Polyphenols and Flavonoids From the n-Butanol Fraction of *Aristolochia longa* Roots by HPLC-DAD-ESI-MS. **1:** Caffeoyl hexoside, **2:** Quinin acid, **3:** Protocatechuic acid hexoside, **4:** 3,5-Di-O-caffeoylquinicacid, **5:** Naringenin–Oglucoside, **6:** Apigenin-8-C-hexoside, **7:** Kaempferol-3-O-glucoside, **8:** Apigenin-7-O-glucoside, **9:** 3,5-Di-*O*-caffeeoylquinic acid.

Discussion

The present study reveals the antioxidant activity of flavonoids extracted from *A. longa*. The phytochemical analysis proved the

Table 4. Polyphenols and Flavonoids of the n-Butanol Fraction of Aristolochia longa Leaves and Roots

Peak	Retention Times	λ max	MH-	Leaves (µg/g DM)	Root (µg/g DM)	Compound
01	3.094	195-261	341	37.89	13.30	Caffeoylhexoside
02	11.712	260	197	31.42	39.11	Quinicacid
03	12.78	217-277	315	235.15	152.41	Protocatechuicacidhexoside
04	16.77	266-330	353	93.83	3.04	3,5-Di-O-caffeoylquinic acid
05	26.37	268-348	593	139.30	-	Kaempferol-3-O-rutinoside
06	27.32	271-332	271	101.58	2.83	Naringenin-O-glucoside
07	28.84	270-336	431	3574.49	40.14	Apigenin-8-C-hexoside
08	30.88	268-349	447	1651.82	61.15	Kaempferol-3-O-glucoside
09	34.012	270-337	431	15.08	33.90	Apigenin-7-O-glucoside
10	36.38	273-327	515	223.09	63.61	3,5-Di-*O*-caffeoylquinic acid
11	40.906	274-341	639	50.92	-	Isorhamnetin-3-*O*-di-glucoside
12	44.78	275-333	593	37.15	-	Apigenin 6,8-di-C-glucoside

presence of bioactive compounds such as tannins, flavonoids. In literature, the presence or absence of natural products are dependent on the polarity of the solvent used.[12] In addition, the yields extraction obtained in this study can be depends on many parameters, including the solvent used, composition of plant material, extraction method. The result of total phenolic and flavonoids content showed that the leaves methanolic extract and leaves n-butanol fraction have the highest amount than roots. According to previous studies on the total phenolic content in some other varieties of *Aristolochia albida,* total phenolic content in ethanolic extract of leaves was found to be 1.88 mg GAE/g DW[25]. Also, the study conducted by Merouani et al[26] showed that the leaves methanolic extract of *A. longa* has the highest amount of flavonoids (52.3 7µg CE/mg DW). In contrary, the studies carried by Djeridane et al[27] showed that the flavonoids content of roots was 0.81 mg CE/g DW of plant. The variation in the total phenolic and flavonoids contents might be attributed to the geographical factors as well as the different cultivation methods, growing seasons, geographic origin and differences in analytical methods.[16,20] However, previous studies demonstrated that the species of genus *Aristolochiacae*[26,28] rich in phenolic compound such as alkaloids, flavonoids, tannins, coumarins, which are responsible for many biological activities including the anticancer, antioxidant and antimicrobial activities. Phenolic compounds are very important because of their action to scavenge the free radicals in the human body and to help maintaining healthy body by scavenging or removing the reactive oxygen species.[20] Several phenolic compounds of plants are known to be responsible for the radical scavenging and antioxidant activities. The natural antioxidants generally function as free radical scavengers and chain breakers, quenchers of singlet-oxygen formation and complexes of pro-oxidant metal ions.[21] The antioxidant capacity and reducing power assay was observed by,[26] where the DPPH radical scavenging activity and reducing capability to convert the potassium ferricyanide (F^{+3}) complex to form potassium ferrocyanide (Fe^{+2}) in the leaves methanolic extract of *A. longa* were: IC50 = 55.04 ± 1.29 µg/mL and EC_{50}=0.2±0.019 mg/mL. This difference might be to the concentration of antioxidant compounds (specially flavonoids) in the extract.

The HPLC analysis of leaves n-butanol fraction, revealed the presence of two major bioactive polyphenolic flavonoids (kaempferol and apiginin) which explained heir highest scavenging ability. The antioxidant properties of flavonoids are widely known.[29,30] Many flavonoids compounds (flavones, flavonols, flavononesand catechin) have antioxidant activities such as peroxynitrite scavenging ability, metal chelating and free radical scavenging capacities. Flavonoids have strong antioxidant properties, which might be due to their ability to chelate transition metal ions, such as Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+}, catalyse electron transport, and scavenge free radicals.[31] Previous studies have shown that kaempferol, some glycosides of kaempferol and several kaempferol-including plants have antioxidant activity not only *in vitro*, but also *in vivo*.[32,33] Various studies showed that Kaempferol has an important anti-inflammatory activity. It can inhibit TNF-α activity, IL-1β and IL-8 expression.[34-36] On the other hand, a number of biological effects of Apigenin innumerous mammalian systems *in vitro* as well *in vivo* are related to its antioxidant and antigenotoxic effects and its role in scavenging free radicals.[37,38] In addition, several properties have been attributed to Apigenin and its derivative, including antioxidant, anti-inflammatory and anti-carcinogenic effects.[39,40]

Conclusions

In view of the result found, *A. longa* leaves marked a high level of flavonoids compared to the roots level. In addition, the same part of the plant exhibited strong antioxidant abilities. These data suggest that *A. longa* can be used as a precious source of antioxidant compounds to prevent different diseases such as, cancer, Alzheimer etc.

Authors' Contributions

BM, ATT and PS designed the study. SA performed the experiments. MM prepared the samples and analysed the fractions composition .SA wrote the paper with input from all authors..

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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References

1. Neinhuis C, Wanke S, Hilu KW, Muller K, Borsch T. Phytogeny of Aristolochiacae based on parsimony, Likelihood and Bayesian analyses of trnL-trnF. Plant Syst Evol. 2005;250:7-26.2. doi:10.1007/s00606-004-0217-0.
2. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. Med Res Rev. 2003;23(4):519-534. doi:10.1002/med.10033.
3. Lattanzio V, Kroon PA, Quideau S, Treutter D. Plant phenolics – secondary metabolites with diverse functions. In: Lattanzio V, Daayf F, Lattanzio V, eds. Recent Advances in Polyphenol Research. Oxford: Wiley-Blackwell; 2009. doi:10.1002/9781444302400.ch1
4. Pacheco AG, Machado de Oliveira P, Piló-Veloso D, Flávio de Carvalho Alcântara A. 13C-NMR data of diterpenes isolated from *Aristolochia* species. Molecules. 2009;14(3):1245-1262. doi:10.3390/molecules14031245.
5. Benarba B, Pandiello A, Elmallah A. Anticancer activity, phytochemical screening and acute toxicity evaluation of an aqueous extract of *Aristolochia longa* L. Int J Pharm Phytopharm Res. 2016;6(1):20-26. doi:10.24896/eijppr.2016614.
6. Heinrich M, Chan J, Wanke S, Neinhuis C, Simmonds MS. Local uses of *Aristolochia* species and content of nephrotoxic aristolochic acid 1 and 2--a global assessment based on bibliographic sources. J Ethnopharmacol. 2009;125(1):108-144. doi:10.1016/j.jep.2009.05.028.
7. Falcone Ferreyra ML, Rius SP, Casati P. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci. 2012;3:222. doi:10.3389/fpls.2012.00222.
8. Croteau R, Kutchan TM, Lewis NG. Natural products (secondary metabolites). In: Buchanan BB, Gruissem W, Jones RJ, eds. Biochemistry and Molecular Biology of Plants. Rockville, MD : American Society of Plant Physiologists, 2000.
9. Sharma V, Janmeda P. Extraction, isolation and identification of flavonoid from *Euphorbia neriifolia* leaves. Arab J Chem. 2017;10(4):509-514. doi:10.1016/j.arabjc.2014.08.019.
10. Njoku V, Obi C. Phytochimical constituents of some selected medicinal plants. Afr J Pure Appl Chem. 2009;3(11):228-233.
11. Sabri FZ, Belarbi M, Sabri S, Alsayadi Muneer MS. Phytochemical screening and identification of some compounds from Mallow. J Nat Prod Plant Resour. 2012;2(4):512-516.
12. Alorkpa EJ, Boadi NO, Badu M, Saah SA. Phytochemical screening, antimicrobial and antioxidant properties of assorted *Carica papaya* leaves in Ghana. J Med Plants Stud. 2016;4(6):193-198.
13. Feknous S, Saidi F, Mohamed- Said R. Extraction, characterization and identification of some active secondary metabolites of lemon balm (*Melissa officinalis* L). Revue "Nature and Technology". A-sciences Fundamentals and Engineering. 2014;11:7-13.
14. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16(3):144-158.
15. Bougandoura N, Bendimerad N. Evaluation de l'activité antioxydante des extraits aqueux et méthanolique de *Satureja calamintha* ssp. *Nepeta* (L.) Briq. Nature & Technology. 2013;9:14-19.
16. Kim DO, Chun OK, Kim YJ, Moon HY, Lee CY. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric Food Chem. 2003;51(22):6509-6515. doi:10.1021/jf0343074.
17. Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese Society of Nutrition and Dietetics. 1986;44(6):307-315. doi:10.5264/eiyogakuzashi.44.307.
18. Waterman PG, Mole S. Analysis of Phenolic Plant Metabolites. Oxford: Blackwell Scientific Publications; 1994.
19. Bello IA, Ndukwe GI, Audu OT, Habila JD. A bioactive flavonoid from *Pavetta crassipes* K. Schum. Org Med Chem Lett. 2011;1(1):14. doi:10.1186/2191-2858-1-14.
20. Hue SM, Boyce AN, Somasundram C. Antioxidant activity, phenolic and flavonoid contents in the leaves of different varieties of sweet potato (*Ipomoea batatas*). Aust J Crop Sci. 2012;6(3):375-380.
21. Rashmika PP, Manish PP. Evaluation of antioxidant activity of *Corchorus aestuans* Linn leaves extracts. Int Res J Pharm. 2012;3(7):233-238.
22. Sreejayan N, Rao MN. Free radical scavenging activity of curcuminoids. Arzneimittelforschung. 1996;46(2):169-171.
23. Assefa AD, Ko EY, Moon SH, Keum YS. Antioxidant and antiplatelet activities of flavonoid-rich fractions of three citrus fruits from Korea. 3 Biotech. 2016;6(1):109. doi:10.1007/s13205-016-0424-8.
24. Barros L, Ferreira MJ, Queirós B, Ferreira ICFR, Baptista P. Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem. 2007;103(2):413-419. doi:10.1016/j.foodchem.2006.07.038.
25. Guinnin FFD, Sacramento IT, Ategbo JM, Agbangnan CDP. Physico-chemical composition and radicalscavenging activity evaluation of the extracts of Aristolochia albida Duch. (*Aristolochiaceae*) of Benin. J Appl Biosci. 2016;107(1)10460-10470. doi:10.4314/jab.v107i1.11.
26. Merouani N, Belhattab R, Sahli F. Evaluation of the biological activity of *Aristolochia longa* L. extracts. Int J Pharm Sci Res. 2017;8(5):1978-1992. doi:10.13040/ijpsr.0975-8232.
27. Djeridane A, Yousfi M, Nadjemi B, Maamri S, Djireb F, Stocker P. Phenolic extracts from various Algerian plants as strong inhibitors of porcine liver carboxylesterase. J Enzyme Inhib Med Chem. 2006;21(6):719-726. doi:10.1080/14756360600810399.
28. Jegadeeswari P, Daffodil ED, Mahan VR. Quantification of total phenolics, flavonoid and in vitro antioxidant activity of *Aristolochia bracteata* Retz. Int J Pharm Pharm Sci. 2014;6(1):747-752.
29. Pietta PG. Flavonoids as antioxidants. J Nat Prod. 2000;63(7):1035-1042.doi:10.1021/np9904509.
30. Rice-Evans C. Flavonoid antioxidants. Curr Med Chem. 2001;8(7):797-807. doi:10.2174/0929867013373011.
31. Kaur P, Arora S. Polyphenols of *Caeselpiniaceae*. J Chin Clin Med. 2010;5(5):282-290.
32. Aniya Y, Koyama T, Miyagi C, et al. Free radical scavenging and hepatoprotective actions of the medicinal herb, *Crassocephalum crepidioides* from the Okinawa Islands. Biol Pharm Bull. 2005;28(1):19-23. doi:10.1248/bpb.28.19.
33. Kampkötter A, Gombitang Nkwonkam C, Zurawski RF, et al. Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism *Caenorhabditis elegans*. Arch Toxicol. 2007;81(12):849-858. doi:10.1007/s00204-007-0215-4.
34. Kowalski J, Samojedny A, Paul M, Pietsz G, Wilczok T. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1beta and tumor necrosis factor-alpha genes in J774.2 macrophages. Pharmacol Rep. 2005;57(3):390-394.
35. Lee S, Kim YJ, Kwon S, et al. Inhibitory effects of flavonoids on TNF-alpha-induced IL-8 gene expression in HEK 293 cells. BMB Rep. 2009;42(5):265-270. doi:10.5483/bmbrep.2009.42.5.265.
36. Rao YK, Fang SH, Tzeng YM. Antiinflammatory activities of flavonoids and a triterpene caffeate isolated from *Bauhinia variegata*. Phytother Res. 2008;22(7):957-962. doi:10.1002/ptr.2448.
37. Liu RH. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr. 2004;134(12 Suppl):3479s-3485s. doi:10.1093/jn/134.12.3479S.
38. Ali F, Rahul, Naz F, Jyoti S, Siddique YH. Protective effect of apigenin against N-nitrosodiethylamine (NDEA)-induced hepatotoxicity in albino rats. Mutat Res Genet Toxicol Environ Mutagen. 2014;767:13-20. doi:10.1016/j.mrgentox.2014.04.006.
39. Škerget M, Kotnik P, Hadolin M, Hraš AR, Simonič M, Knez Ž. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 2005;89(2):191-198. doi:10.1016/j.foodchem.2004.02.025.
40. Dimitrios B. Sources of natural phenolic antioxidants. Trends Food Sci Technol. 2006;17(9):505-512. doi:10.1016/j.tifs.2006.04.004.