



Received on 09 October, 2016; received in revised form, 27 December, 2016; accepted, 31 December, 2016; published 01 May, 2017

## EVALUATION OF THE BIOLOGICAL ACTIVITY OF *ARISTOLOCHIA LONGA* L. EXTRACTS

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### Keywords:

*Aristolochia longa* L., Polyphenols, Antioxidant activity, Antibacterial activity, Antifungal activity, Anti-inflammatory activity

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**ABSTRACT:** *Aristolochia longa* L. (Aristolochiaceae) is a native plant of Algeria used in traditional medicine. This study was devoted to the determination of polyphenols, flavonoids, and tannins contents of *A. longa* L. extracts. Extracts were prepared from aerial parts (stems and leaves), fruits and tubers by using various solvents with different polarities such as acetone, methanol and distilled water. Acetone extracts from the aerial parts presented the highest contents of polyphenols (525.43±29.6 µg/mg) followed by fruit aqueous extract (518.54±14.93 µg/mg), while the aerial parts methanol extract shown the highest flavonoid content (52.37±0.94 µg/mg) and exhibited the highest antioxidant capacity of DPPH and reducing power (55.04 ±1.29 µg/mL and 0.2±0.019 mg/mL, respectively), therefore the aerial parts acetone extract showed the highest antioxidant capacity in the β-carotene bleaching inhibition test with 57%. For antimicrobial activity, the fruit methanol extract was too efficient against the bacterial strains tested, whereas no effect was observed when these extracts were tested against fungi. The protein denaturation was found in the aerial parts acetone extract to be 78.35±6.18% followed by fruit methanol extract, 68.04±4.72% at the dose 500 µg/mL, with regards to standards diclofenac sodium. These preliminary results could be used to justify the traditional use of this plant and its bioactive substances could be exploited for therapeutic purposes.

**INTRODUCTION:** Species belonging to the genus *Aristolochia* have often been reported as important medicinal plants in ethnobotanical studies. They are widely distributed in practically all continents except Australia, a continent for which only few species are known <sup>1</sup>. *Aristolochia* species contain secondary metabolites that are important natural toxins and traditional medicines <sup>1</sup>.

The data obtained by Heinrich et al., (2009) demonstrate the worldwide importance of members of the genus in practically all regions where *Aristolochia* species are found.

*Aristolochia longa* L. (Aristolochiaceae) widely distributed in Algeria <sup>2</sup> and locally known as ‘Beroustoum’, is a species commonly used in Algerian traditional medicine. Tubers of this plant were used such as astringent, antirheumatic, antitumor, anti-inflammatory and antiseptic <sup>3,4</sup>.

The aim of this study was to assess the biological activity of the different extracts of *Aristolochia longa* L., to determine the effect of different

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extraction solvents on these activities and to conclusion the most effective extract.

## MATERIALS AND METHODS:

### Plant material and extracts preparation:

*Aristolochia longa* L. was collected in May 2011, 80 km North of Setif (Algeria). A voucher specimen was deposited in the Nature and Life Sciences Faculty herbarium (University F.A.Setif1, Algeria)

The aerial parts (stem and leaves), fruits and tubers were shadow-dried and pulverized to dry powder. All chemicals were purchased from Sigma.

Three extracts (acetone, methanol and water) were prepared from aerial parts (stem and leaves), fruits and tubers. 20 grams of the different part of dried plant material were crushed and extracted for 48 h with 100 ml of 80% (v/v) aqueous methanol at room temperature. Second successive extraction with 50 ml of the same hydro alcoholic solution was carried out at room temperature for 24 h. After removal of methanol under reduced pressure in a rotary evaporator at 40 °C, the remaining aqueous solution of the extraction is defatted twice with petroleum ether to remove lipids. Then, the lyophilized solution is extracted with ethyl acetate in presence of aqueous solution with 20% ammonium sulfate, and 2% of ortho-phosphoric acid solution. The ethyl acetate fraction is dried with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The dried precipitate was dissolved in 5 ml of absolute methanol and kept at - 10 °C<sup>5</sup>.

The same parts of plant were extracted with acetone in a Soxhlet apparatus within a period of 6 h. The solvent was removed under vacuum and the crude acetone extract obtained. For the preparation of water extracts, the plant, 20g, was extracted with boiling distilled water (250ml) for 10min. the extract remained in the warm water for 15 min and then filtered and concentrated in rotary evaporator<sup>6</sup>.

**Plant extract yield (EY):** The yield of the extraction was calculated from the following equation

$$(W_1/W_2) \times 100$$

Where,  $W_1$  is the weight of extract after evaporation of solvent and  $W_2$  is the dry weight of the plant sample.

### Determination of antioxidant components:

**Total phenol content:** In this study, we've estimated total phenolics using the Folin– Ciocalteu reagent as previously described<sup>7</sup>. Samples were incubated at room temperature for 2 h, the absorbance of all samples were measured at 765 nm against a methanol blank using a spectrophotometer. The standard calibration curve was obtained using gallic acid. Total phenol content was expressed as  $\mu\text{g}$  of gallic acid equivalents (GAE) / mg of dry extracts.

**Flavonoids content:** The determination of total flavonoids content was conducted according to the  $\text{AlCl}_3$  method<sup>4</sup>. The absorbance of mixture was measured at 430 nm after 30 min of incubation at room temperature. Results were expressed as  $\mu\text{g}$  of quercetin equivalents (QE) / mg of extract.

**Flavones and flavonols content:** Flavones and flavonols were estimated according to the protocol developed by Kosalec et al.<sup>8</sup>, where the absorbance of mixture was measured at 415 nm after 30 min of incubation at room temperature. The content of flavones and flavonols was expressed as  $\mu\text{g}$  of quercetin equivalents (QE) / mg extract.

**Tannins content:** For the determination of tannin content in extracts, a method proposed by Bate-Smith<sup>9</sup> was followed. Results were expressed as microgram of tannin equivalent per mg of extract (TAE/ mg extract). All tests were carried out in triplicate.

**$\beta$ - carotene and lycopene contents:**  $\beta$ - carotene and lycopene contents were simultaneously determined by a spectrophotometric method<sup>10</sup>. The dried methanolic extract (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6 v/v) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm.

Contents of  $\beta$ -carotene and lycopene were calculated according to the following equations:

$$\text{Lycopene (mg/100 ml)} = - 0.0458 A_{663} + 0.372 A_{505} - 0.0806 A_{453}.$$

$$\beta\text{- carotene (mg/100 ml)} = 0.216 A_{663} - 0.304 A_{505} + 0.452 A_{453}.$$

The assays were carried out in triplicate; the results were mean values  $\pm$  standard deviations and expressed as mg of carotenoid/g of extract<sup>11,12</sup>.

**Antioxidant activity:** Three different *in vitro* tests were carried using solutions prepared by serial dilution: scavenging effects on DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals, reducing power (measured by ferricyanide Prussian blue assay), inhibition of  $\beta$ -carotene bleaching.

**DPPH radical scavenging assay:** The potential antioxidant activity of plant extracts was assessed on the basis of the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the previous described procedures. Different concentrations of test samples were prepared. The reaction mixtures, consisting of 1ml test sample and 1ml methanolic solution of DPPH,  $6.10^{-5}$  M, were incubated for 30 min at room temperature in the dark and absorbance was measured at 515 nm. Methanol was used to zero the spectrophotometer. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{The percentage inhibition of the DPPH radical (\%)} \\ = \frac{A_0 - A_1}{A_0} \times 100$$

Where,  $A_0$  is the absorbance of the control at  $t = 0$  min and  $A_1$  is the absorbance of the sample.

The  $IC_{50}$  ( $\mu\text{g/mL}$ ) values denote the concentration of each sample required to give 50% of the optical density shown by the control. A lower  $IC_{50}$  value corresponds to a higher antioxidant activity of sample. BHT (butylated hydroxy toluene) was used as a standard antioxidant<sup>13</sup>.

**Reducing power assay:** Reducing power was evaluated by the capacity to convert  $\text{Fe}^{3+}$  into  $\text{Fe}^{2+}$ . The power iron reducer ( $\text{Fe}^{3+}$ ) in extracts is determined according to the method described by Oyaizu (1986)<sup>14</sup>. Briefly, a solution of 2.5 ml of phosphate buffer (0.2 M pH = 6, 6) and 2.5 ml of potassium ferri-cyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] (1%) were added to 1ml of the extract in different concentrations. Reaction mixture was incubated at 50 °C for 20 min and then 2.5 ml of trichloroacetic acid (10%) was added and centrifuged for 10 min. From the upper layer, 2.5 ml was mixed with 2.5 ml of distilled water and 0.5 ml of  $\text{FeCl}_3$  (0.1%).

Finally, the absorbance was measured at 700 nm against a blank. Quercetin and BHA (butylated hydroxy anisole) were used as a control.

A higher absorbance indicates a higher reducing power.  $EC_{50}$  value (mg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis<sup>15, 16, 17</sup>.

**$\beta$ - Carotene– linoleic acid assay:** In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation<sup>18</sup>. In brief, 0.5 mg  $\beta$ -carotene in 1 ml chloroform was mixed with 25  $\mu\text{l}$  of linoleic acid and 200 mg of Tween40. The chloroform was evaporated under vacuum at 45 °C, then 100 ml distilled water saturated with oxygen was added and the resulting mixture was vigorously stirred.

The emulsion obtained was freshly prepared before each experiment. An aliquot (2.5 ml) of the  $\beta$ -carotene/ linoleic acid emulsion was transferred to tubes containing 0.5 ml of each sample at different concentrations. The tubes were immediately placed in water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. A control consisted of 0.5 ml of distilled water instead of the sample solution. BHA was used as positive standard<sup>19, 20</sup>. Tests were carried out in triplicate. The relative antioxidant activity was calculated according to the following formula:

$$\text{Antioxidant activity (\%)} = 1 - \frac{(\text{At}_0 - \text{At}_{120})_{\text{test}}}{(\text{At}_0 - \text{At}_{120})_{\text{control}}} \times 100$$

$\text{At}_0$ : absorbance at time  $t = 0$ .

$\text{At}_{120}$ : absorbance at time  $t = 120$ min.

***In vitro* anti-inflammatory activity:** Anti-inflammatory activity of *Aristolochia longa* extracts was evaluated by protein denaturation method<sup>21</sup>.

In this experiment, four solutions were prepared : test solution consist of 0.45 ml of BSA (bovine serum albumin) (5% w/v) (aqueous solution) and 0,05 ml extract solution (500  $\mu\text{g/mL}$ ), test control solution consist of 0.45 ml of BSA (5% w/v)

(aqueous solution) and 0.05 ml distilled water, product control solution consist of 0.45 ml distilled water and 0.05 ml of extract solution (500 µg/mL), standard solution consist of 0.45 ml Of BSA (5% w/v) (aqueous solution) and 0.05 ml diclofenac sodium (500 µg/mL), each previous solution contains 0.5 ml. All above solutions were adjusted to pH 6.3 using 1N hydrochloric acid. The samples were incubated at 37 °C for 20 min and the temperature was increased to keep the samples at 57 °C for 3 min. After cooling, 2.5 for phosphate buffer saline was added to above solutions the absorbance was measured at 416 nm. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\text{Percent inhibition \%} = 1 - \frac{\text{O.D of the test solution} - \text{OD of product control}}{\text{OD of test control}} \times 100$$

The control represents 100% protein denaturation. The results were compared with diclofenac sodium (500 µg/mL). Each experiment was done in triplicate and the average was taken.

#### Anti- microbial activity:

**Anti-bacterial activity:** Extracts were tested against the reference strains for their inhibitory activity, using two methods: agar diffusion method<sup>22</sup> and the microdilution method (minimum inhibitory concentrations (MIC)<sup>23</sup>.

The anti-bacterial activity of the extracts was tested against four aerobic reference bacterial strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 10876.

The bacteria were grown on Mueller Hinton agar. Gentamycin was used as positive control since it is commonly used as antibiotic against gram positive and gram negative bacterial sp.

A volume (20 mL) of each medium was poured into 90 mm diameter Petri dishes. The bacteria used in the tests were obtained from 24 h cultures and suspended in a sterile saline solution to obtain an inoculum that has a concentration of approximately 10<sup>8</sup> CFU/mL (or 0,5 Mac Farland). The agar plate containing the appropriate medium was spread with the inoculum containing 10<sup>8</sup> CFU/mL.

**Anti-fungal activity:** The moulds (*Aspergillus flavus* NRRL 391, *Aspergillus niger* 2CA 936) were separately inoculated on to PDA medium and incubated for 7 days at ambient temperature until sporulation was complete. The spores were harvested with 10 ml sterile distilled water and adjusted to contain approximately 10<sup>6</sup> spores/mL. A cell suspension of yeast (*Candida albicans* ATCC1024) was prepared in sterile distilled water and adjusted to contain approximately 10<sup>6</sup> spores/mL. 1ml of the spores was added to plates containing PDA medium, while the yeast suspension was added to plates containing Saboureaux + chloramphenicol + actidione<sup>6</sup>.

In both activities (Anti-bacterial activity and Anti-fungal activity), the sterilized discs (6 mm in diameter) were impregnated with 10 µl of the extract and then placed on to agar plates. After incubation at 37 °C for 18–24 h for bacteria, at 30 °C for 24–48 h for yeasts and 10 days at room temperature for moulds, the diameters of inhibition zones were measured in mm. Gentamycin (10 µg), and clotrimazole (50 µg) were used as positive controls for bacteria, yeast and moulds, respectively. Pure dimethyl sulfoxide (DMSO) 10 µl was injected as the negative control. All the experiments were performed in triplicate.

**Minimum inhibitory concentration (MIC):** Minimum inhibitory concentration (MIC) was determined using a common broth microdilution method, in 96 multiwell microtiter plates, in triplicate<sup>23</sup>.

In a first step 50 µl of Mueller Hinton broth were distributed from the second to the twelfth test tubes. Dry extracts were initially dissolved in 10% of dimethyl sulfoxide (DMSO) and then in Mueller Hinton Broth, to reach a final concentration of 200 mg/mL for methanol and acetone extracts and 400 mg/mL for water extract; 100 µl of these suspensions were added to the first test well of each microtiter line, and then 50 µl of scalar dilution were transferred from the second to the ninth well. The 10<sup>th</sup> well was considered as growth control, since no extracts solutions were added. Then, 50 µl of a microbial suspension (10<sup>5</sup> colony forming units, CFU/mL), obtained from an overnight growth at 37 °C, were added to each well.

The final concentration of the extracts adopted to evaluate the anti-bacterial activity was included from 100 mg/mL (first well) to 0.390 mg/mL (ninth well) and for water extracts the anti-bacterial activity was included from 200 mg/mL (first well) to 0.780 mg/mL (ninth well). Plates were incubated for 18h at 37 °C. After incubation period, the vials were checked for turbidity and the lowest concentrations of the extract showing no turbidity (no growth) were regarded as the MIC of the test substance<sup>24, 25</sup>.

**Statistical analysis:** The results are expressed as the mean value  $\pm$  standard deviation. One-way analysis of variance followed by the Tukey test was performed to assess differences between groups. Differences were considered significant at  $p < 0.05$ . Statistical analyses were performed with the aid of the software GraphPad Prism 5®.

## RESULTS AND DISCUSSION:

**Extracts yields:** The extraction yields were obtained after removal of solvent, which ranged from 1.45% to 10.05% (w/w) and were influenced by several parameters, including chemical composition and physical characteristics of the plant material<sup>26</sup>. In our study, the results showed that the aqueous extracts gave us highest yields (**Table 1**). For example, the highest yield in extracts was achieved by the aerial part aqueous extract, where it had considerable proportion of phenols content. The aqueous extraction is carried out by high temperature decoction for 10 min, in fact Su et al., reported that aqueous extraction efficiency increases with temperature<sup>27</sup>. This explains that water high-temperature causes disruption of cells facilitating the penetration of the solvent and solubilizing molecules<sup>28</sup>. Martins et al.,<sup>29</sup> reported that the decoction presented the highest concentration of phenolic compounds (phenolic acids and flavonoids) followed by infusion and hydroalcoholic extract.

Koruthu et al., said that the water soluble phenolics are only important as antioxidant compounds<sup>30</sup>. It has been reported that the solvent polarity highly affects extraction rate and antioxidant activity of plant extracts<sup>31</sup>, and the efficiency of the extraction depends on many parameters, including the extraction time and temperature, the volume and type of the solvents used<sup>32</sup>.

**Total phenolics content:** Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants<sup>7</sup>. It has been suggested that polyphenolic compounds have inhibitory effect on mutagenesis and carcinogenesis in humans, when ingested at up to 1g daily from a diet rich in fruits and vegetables<sup>4</sup>.

The content of total polyphenols, flavonoids and tannins in aqueous, methanol and acetone extracts of *Aristolochia longa* L. were determined following the Folin- Ciocalteu procedure, which is considered as the best method for total phenolics (including tannins) evaluation, the aluminum trichloride, and the hemoglobin precipitation method respectively. The results showed that the total phenols were characterized by highest values compared with other ingredients in all extracts used (**Table 1**), but the highest amounts of total phenolics were found in aerial parts acetone extract (525.43 $\mu$ g GAE/mg extract), and in fruit aqueous extract (518.54 $\mu$ g/mg).

The total phenolic content were calculated using the following linear equation based on the calibration curve of gallic acid:  $A=0,089X+0.0199$  ( $R^2=0.9995$ ),

Where, A is the absorbance and X is the amount of gallic acid in  $\mu$ g.

**Flavonoids content:** The content of flavonoids expressed in quercetin equivalents varied from 4.86- 52.37  $\mu$ g/mg, the highest amounts were found in aerial parts methanol extract and aerial parts acetone extract (52.37 and 37.54  $\mu$ g/mg respectively), whereas the other extracts contained lower amounts.

The calibration curve of quercetin:

$$A=0.0358X+0.0858 \quad (R^2=0.9984),$$

**Flavones and flavonols content:** The concentration of flavones and flavonols, expressed in microgram of quercetin equivalents per ml of extract varied from 21.64- 85.37 $\mu$ g/mL, according to calibration curve:  $A=0.059X-0.0027$  ( $R^2=0.999$ )).

We also noticed that the aqueous extracts contained remarkably lower amounts of these compounds in comparison with acetone and methanol extracts where we observed the highest amounts (85.37 µg/mL in aerial parts methanol extract and 75.45 µg/mL in aerial parts acetone extract).

Previously, Djeridane et al., reported the following results from roots methanol extract of *Aristolochia longa* L.<sup>4</sup>: total phenolics 1.47±0.02 mg GAE/g dw, flavonoids content 0.81±0.02 mg RE/g dw, flavonols content 0.41±0.002 mg QE/g dw, and also in other studies<sup>33</sup>, total phenolic content in *A. longa* aqueous extract of roots was found to be 6.07 ± 0.12 mg GAE/g, but our extract contain higher levels, this could be explained by the change of the plot location.

**Tannins content:** Determining the tannins content is made by the hemoglobin precipitation test. The content of tannin expressed in µg tannic acid equivalents (TAE) per mg of extract.

$$A = -0.01X + 0.8092 \quad (R^2 = 0.9887),$$

In regards to the content of tannin we noticed that tuber methanol extract have the highest amount (206.93 µg TAE/mg).

According to Seidel, water and methanol are both polar solvents which extract particularly glycosylated flavonoids and tannins<sup>34</sup>, and we could interpret these results by the occurrence of different types of tannins. It is possible to divide the tannins into 2 groups according to their structure: condensed tannins and hydrolysable tannins<sup>35</sup>. We conclude that the difference in results is due to the type of the solvent and the part of the plant.

Phenolic compounds possess scavenging ability due to their hydroxyl groups and are known to be powerful antioxidant<sup>12</sup>. Phenolic compounds such as tannins and flavonoids are considered to be the major contributors to the antioxidant capacity of plants. Some of diverse biological activities of plants, such as antibacterial activity, may also be related to phenolic compounds<sup>32</sup>.

**β-carotene and lycopene content:** β-carotene and lycopene were only found in vestigial amounts, the first compound amount varied from 0.023 -1.036 µg/mg. Aerial parts methanol and acetone extracts have the highest content 1.036 and 0.947 µg/mg respectively, whereas lycopene was present in trace amounts in these extracts (0.0065 -0.690 µg/mL respectively).

**TABLE 1: ANTIOXIDANTS CONTENTS OF ARISTOLOCHIA LONGA L. EXTRACTS**

Extracts	Total phenolics (µg GAE/mg extract)	Flavonoids (µg QE/mg extract)	Flavones and Flavonols (µg QE/mg extract)	Tannins (µg TAE/mg extract)	β-Carotene (µg/mg extract)	Lycopene (µg/mg extract)	Extract yields (%)
Aerial parts aqueous extract (AAE)	396.88±8.86	9.92±0.23	27.40±0.71	54.4±2	0.393±0.004	0.0213±0.001	8.65
Fruit aqueous extract (FAE)	518.54±14.93	5.81±0.15	21.64±1.19	14.14±0.09	0.023±0.001	0.015±0.001	10.05
Tuber aqueous extract (TAE)	293.82±9.90	4.86 ± 0	23.16±0.71	21.2±1.52	0.039±0.001	-	2.7
Aerial parts methanol extract (AME)	132.33±3.77	52.37±0.94	85.37±5.03	99.36±2.78	1.036±0.1	0.305±0.005	3.35
Fruit methanol extract (FME)	260.25±8.49	9.33±0	74.94 ±0.35	155.46±5.10	0.549±0.006	0.0065±0.0005	2.15
Tuber methanol extract (TME)	224.29±13.49	15.42±0.47	62.15±1.91	206.93±4.61	0.638±0.032	-	4.35
Aerial parts acetone extract (AAcE)	525.43±29.6	37.54±0.98	75.45±4.35	132.3±5.12	0.947±0.067	0.690±0.045	6.4
Fruit acetone extract (FAcE)	264.91±4.27	6.13±0.25	37.23±0.23	130.8±3.96	0.88±0.008	0.105±0.005	2.75
Tuber acetone extract (TAcE)	427.31±51.50	7.63±0.19	31.05±1.79	14.98±0.64	0.484± 0.016	0.122±0.002	1.45

Values expressed are means ± S.D. of three parallel measurements.

**Antioxidant activity:**

**DPPH free radical scavenging activity:** DPPH (1,1-diphenyl-2-picrylhydrazyl) is a useful reagent for investigating the free radical-scavenging activities of compounds<sup>36</sup>, and it is a stable free radical that shows a characteristic absorbance at 517 nm, which decreases significantly when exposed to radical scavengers by providing hydrogen atom or electron to be a stable diamagnetic molecule. Upon reduction, solution of DPPH fades from purple to yellow. Thus, a lower absorbance at 517 nm indicates a higher radical scavenging activity of extract<sup>12</sup>.

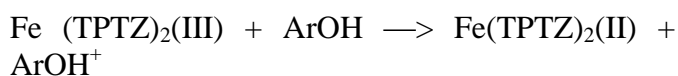
Representing the DPPH radical by Z\* and the donor molecule by AH, the primary reaction is<sup>37</sup>:  
 $Z^* + AH \longrightarrow ZH + A$

The scavenging effects of different concentrations from different extracts of *Aristolochia longa* L. on the DPPH free radical were compared with standard anti-oxidant BHT. The results were expressed as IC<sub>50</sub> (µg/mL) values correspond to the sample concentration providing 50% of antioxidant activity (**Fig. 1**).

As can be seen from the **Table 2** in DPPH assay, the IC<sub>50</sub> values of the antioxidant capacity varied significantly (P < 0.05) from 145.14 to 547.29 µg/mL in comparison with BHT (42.85 µg/mL), however the aerial parts methanol extract scavenging ability (55.04 µg/mL) was found non significant (p>0.05) in comparison with BHT. As it is known, the lower the IC<sub>50</sub> value means the higher the antioxidant capacity of the plant extract. This scavenging activity especially assigned to phenolic compounds i.e phenolic acids and flavonoids<sup>4</sup>, this comment concord with our results because aerial parts methanol extract (who has lower IC<sub>50</sub>) has the highest contents of flavonoids, flavones and flavonols. Our study are in accordance with previous studies which indicate that not only total content, but the type of phenolics and their relative distribution is important for biological activity<sup>38</sup>.

Previous published papers<sup>4</sup> demonstrated that the root methanol extract of *Aristolochia longa* L. have weaker ability to act as antioxidant IC<sub>50</sub>= 90µM/L, This is in accordance with our study since we found a lower IC<sub>50</sub> (514.58 µg/mL).

**Reducing power assay:** Reducing power of a compound indicates its potential antioxidant activity<sup>12</sup>, and it is a mechanism which measures the conversion of a Fe<sup>+3</sup>/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe<sup>2+</sup> was then monitored by measuring the formation of Perl's Prussian blue at 700 nm. In fact, it is widely accepted that higher absorbance at 700 nm is correlated to power reducing<sup>20,37</sup>.



We obtained similar results in reducing power activity (**Table 2**, **Fig. 2** and **Fig. 3**), where the values of EC<sub>50</sub> varied significantly (P < 0.05) from 0.632 to 5.999 mg/mL, but the aerial parts methanol extract showed the highest reducing power activity where was EC<sub>50</sub> (0.2mg/mL) non significant (p>0.05) in comparison with BHA and quercetin (EC<sub>50</sub>= 0.053mg/mL).

**β- Carotene– linoleic acid assay:** The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radical from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate and other free radicals formed in the system<sup>36</sup>. The antioxidant assay using the discoloration of β-carotene is widely used to measure the antioxidant activity of bioactive compounds because β-carotene is extremely susceptible to free radical mediated oxidation of linoleic acid. Furthermore, β-carotene is used as a coloring agent for beverages, and its discoloration would markedly reduce the quality of these products. In this test, β-carotene undergoes rapid discoloration in the absence of antioxidant, which results in a reduction in absorbance of the test solution with reaction time. The presence of antioxidant hinders the extent of bleaching by neutralizing the linoleic free radical formed<sup>19</sup>.

As shown in **Table 2**, all extracts at the same concentration (2 mg/mL) inhibited the oxidation of β- carotene at different degrees (**Fig. 4** and **Fig. 5**), while the aerial parts acetone extract revealed the highest β- carotene bleaching inhibition percentage compared to the other extracts but this activity was

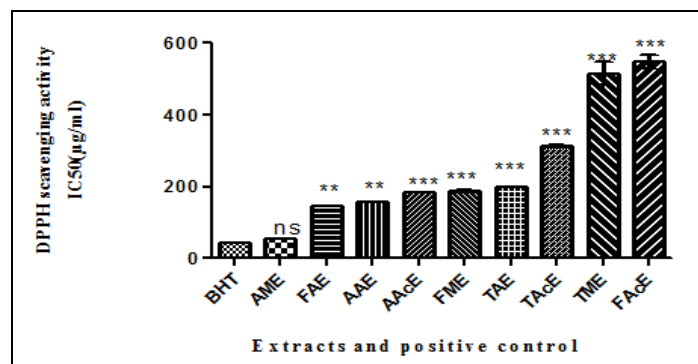
weaker than the activity achieved by BHA (57% versus 84%). The variation in the antioxidant activity could be due to the quantity of polyphenols present in each solvent, but it also varies according to the quality of polyphenols, flavonoids and

tannins. Using solvents with different polarities allow the extraction of a selected group of antioxidants, affecting the antioxidant capacity estimation<sup>39</sup>.

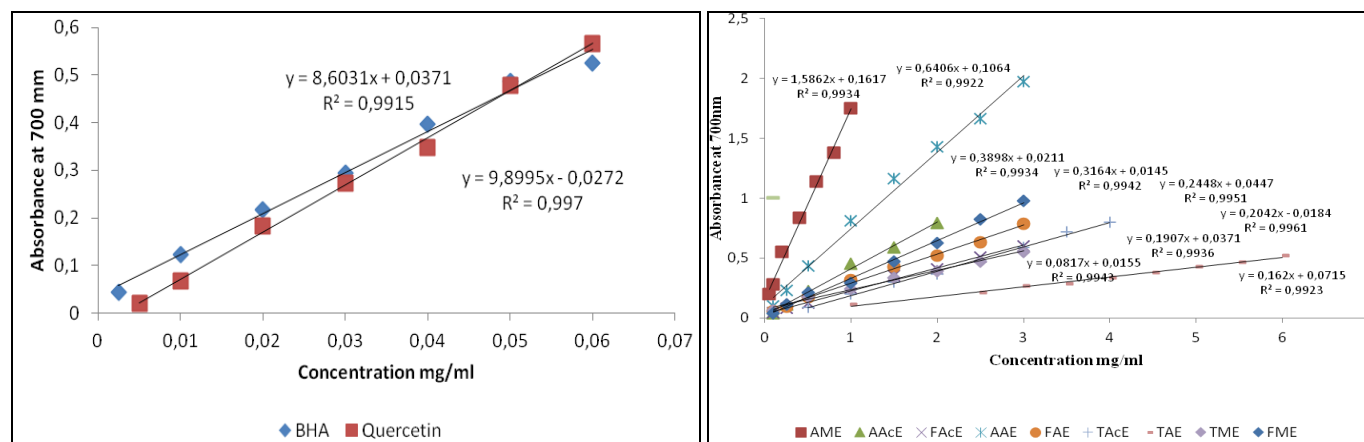
**TABLE 2: IN VITRO ANTIOXIDANT ACTIVITIES OF A. LONGA EXTRACTS**

Extracts	DPPH	Reducing power	b-Carotene bleaching
	IC <sub>50</sub> (µg/mL)	EC <sub>50</sub> (mg/mL)	inhibition%
Aerial parts aqueous extract (AAE)	157.13±0.26**	0.632±0.012***	30±1.73***
Fruit aqueous extract (FAE)	145.15±0.78**	1.862±0.067***	16±1.51***
Tuber aqueous extract (TAE)	198.06±1.7***	5.999±0.075***	12±1.65***
Aerial parts methanol extract (AME)	55.04±1.29 <sup>ns</sup>	0.200±0.019 <sup>ns</sup>	33±1.02***
Fruit methanol extract (FME)	186.21±6.24***	1.535±0.035***	28±2.83***
Tuber methanol extract (TME)	514.58±46.38***	2.645±0.09***	22±2.22***
Aerial parts acetone extract (AAcE)	182.59±1.20***	1.237±0.09***	57±1.79***
Fruit acetone extract (FAcE)	547.29±25.82***	2.427±0.003***	36±3.37***
Tuber acetone extract (TAcE)	311.27±5.83***	2.499±0.137***	23±1.8***
BHT (µg/mL)	42.85±0.15	-	-
BHA (mg/mL)	-	0.053±0.0009	84±5.45
Quercetin (mg/mL)	-	0.053±0.0001	-

Values expressed are means ± S.D. of three parallel measurements. Comparison was realized against BHA, BHT and quercetin; \*  $p < 0.05$  significant difference, \*\*  $p < 0.01$  very significant difference, \*\*\*  $p < 0.001$  extremely significant difference. ns: non significance. IC<sub>50</sub> (µg/mL) values correspond to the sample concentration providing 50% of antioxidant activity. Reducing power was expressed as concentration giving absorbance of 0.500. EC<sub>50</sub> (mg/mL): effective concentration at which the absorbance is 0.5.



**FIG. 1: FREE RADICAL- SCAVENGING CAPACITIES OF A. LONGA L. EXTRACTS AND BHT (AS POSITIVE CONTROL) MEASURED BY DPPH ASSAY.** Values presented are the means of triplicate analysis. \*:  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . ns: non significance.



**FIG. 2: REDUCING POWER ACTIVITY OF A.LONGA L. EXTRACTS AT DIFFERENT CONCENTRATIONS. BHA AND QUERCETIN WERE USED AS POSITIVE CONTROLS. VALUES PRESENTED ARE THE MEANS OF TRIPLICATE ANALYSIS**



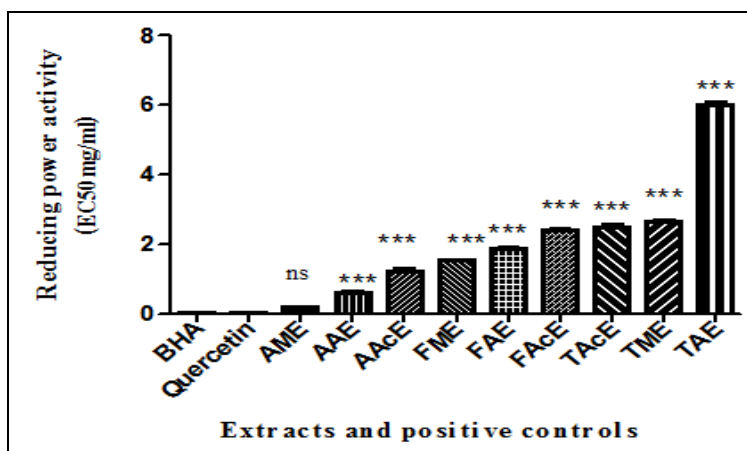


FIG. 3: ANTIOXIDANT CAPACITIES OF *A. LONGA* L. EXTRACT, USING FERRIC REDUCING POWER METHOD. VALUES PRESENTED ARE THE MEANS OF TRIPPLICATE ANALYSIS. Comparison was realized against BHA; \*\*\*:  $p < 0.001$ . ns: non significance

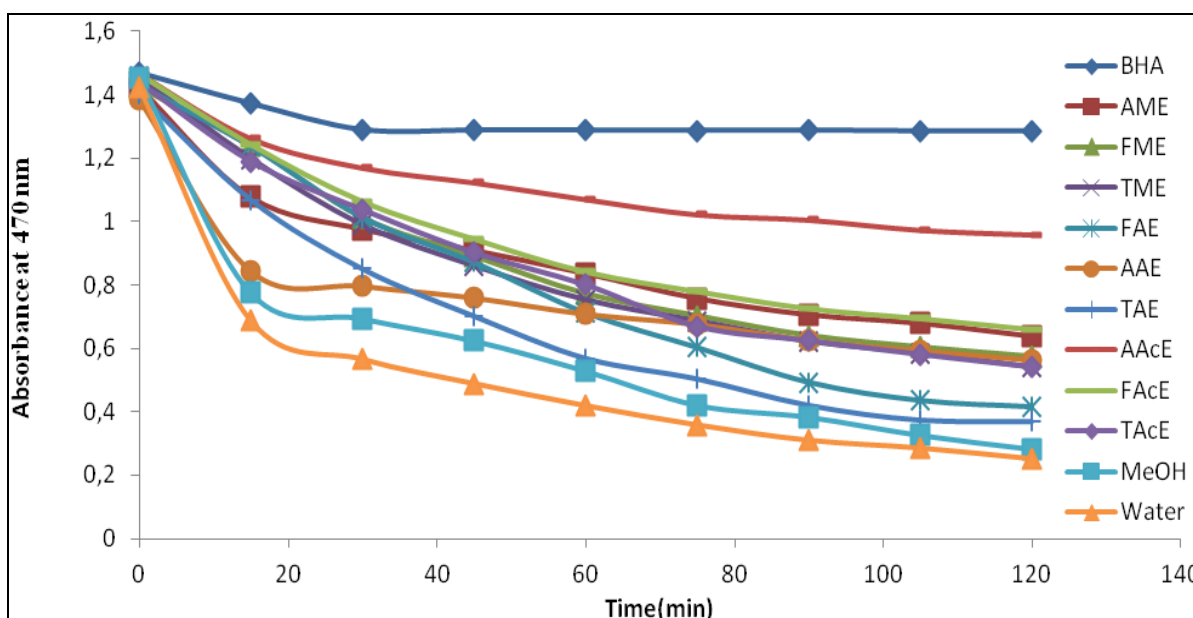


FIG. 4: CHANGE IN ABSORBANCE OF THE  $\beta$ -CAROTENE AT 470 NM IN THE PRESENCE OF *ARISTOLOCHIA LONGA* L. EXTRACTS, BHA AND NEGATIVE CONTROLS (DISTILLED WATER AND METHANOL). VALUES PRESENTED ARE THE MEANS OF TRIPPLICATE ANALYSIS.

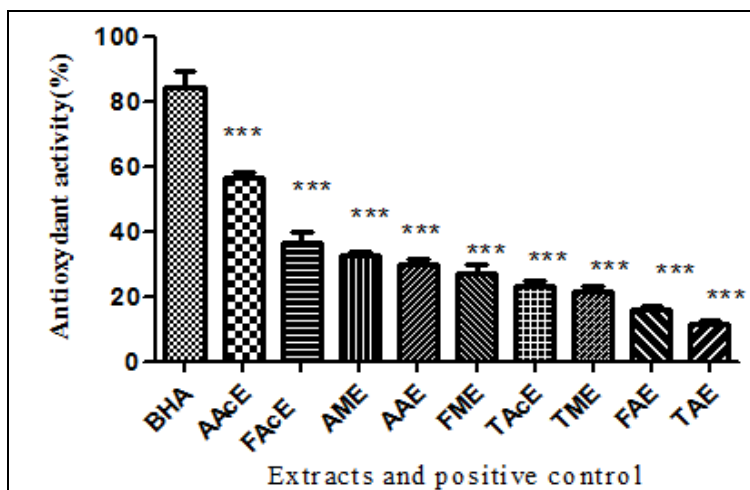


FIG. 5: ANTIOXIDANT ACTIVITY OF *A. LONGA* EXTRACTS AND POSITIVE CONTROL (BHA), MEASURED BY  $\beta$ -CAROTENE BLEACHING ESSAY. Values presented are the means of triplicate analysis. \*\*\*:  $p < 0.001$ .

**In vitro anti-inflammatory activity:**

**Inhibition of protein denaturation method:** The inhibitory effect of different extracts at the concentration 500µg/mL on protein denaturation was showed in **Table 3 (Fig. 6)**. The aerial parts acetone extract exhibited the highest inhibition of protein denaturation (78.35±6.18%), and its effect was found no significant ( $p>0.05$ ) in comparison with the standard of anti inflammation drug, diclofenac sodium showed the maximum inhibition (85.56±1.7%) at the same concentration.

Denaturation of protein is one cause of inflammation<sup>21</sup>. Production of autoantigen in certain arthritic diseases may be due to denaturation of protein<sup>40</sup>. The mechanism of denaturation probably involves alteration I

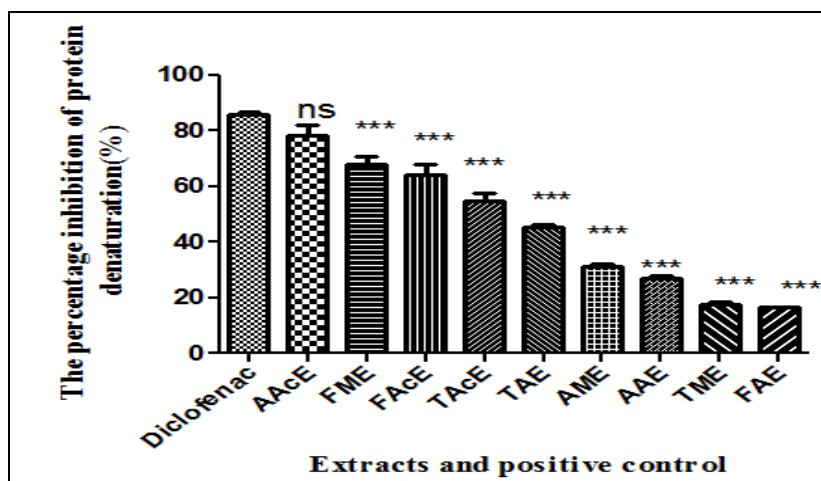
electrostatic hydrogen, hydrophobic and disulfid bonding<sup>41</sup>.

Medicinal plants used in traditional medicine treat anti-inflammatory conditions seem a viable and logical alternative in search of safe and effective anti-inflammatory agents<sup>42</sup>. The anti-inflammatory activity of *Aristolochia* species has been recently described<sup>43</sup>. From the data obtained, successive Soxhlet acetone extract of aerial parts has a highest percentage of inhibition followed by fruit methanol extract. This result was in concordance with the study of Murugan and Parimelazhagan<sup>40</sup> where successive Soxhlet methanol extract of *Osbeckia parvifolia* has the ability to protect the protein membrane from heat and alkali induced protein denaturation comparable to diclofenac.

**TABLE 3: IN VITRO ANTI-INFLAMMATORY ACTIVITY OF A. LONGA EXTRACTS**

Extract (µg/mL)	Water			Methanol			Acetone			Diclofenac Sodium (µg/mL)
	Aerial parts (AAE)	Fruits (FAE)	Tubers (TAE)	aerial parts (AME)	Fruits (FME)	Tubers (TME)	Aerial parts (AAcE)	Fruits (FAcE)	Tubers (TAcE)	
Percentage inhibition of protein Denaturation	26.80*** ±1.78	16.49*** ±0	45.36*** ±1.78	30.92*** ±1.78	68.04*** ±4.72	17.52*** ±1.78	78.35 <sup>ns</sup> ±6.18	63.91*** ±6.43	54.63*** ±4.72	85.56 ±1.78

Values are mean± SD, n=3. Comparison was realized against diclofenac sodium. \* $p<0.05$ , \*\* $p < 0.01$ , \*\*\*  $p<0.001$ ; ns: non significance



**FIG. 6: IN VITRO ANTI-INFLAMMATORY ACTIVITY OF A. LONGA EXTRACTS. COMPARISON WAS REALIZED AGAINST DICLOFENAC SODIUM; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$ . ns: non significance**

**Anti-microbial activity:**

**Anti-bacterial activity:** Plant extracts were tested at various concentrations against gram positive and gram negative bacterial strains. Methanol and acetone extracts were tested from 100 mg/mL, whereas the aqueous extracts were tested from 200 mg/mL. The results are reported in **Table 4**. The

antimicrobial activity of plants is related to their zone of inhibition against some of the pathogenic organisms. The fruit methanol extract (FMA) showed the highest inhibitory effects against *P. aeruginosa* with inhibition zone diameter of 20 mm, followed by *S. aureus* and *B. cereus* with inhibition zones of 18.5 and 15mm respectively,

almost similar results were observed with the acetone extracts from the aerial parts (AAcE) (*P. aeruginosa* 19mm, *S. aureus* 14mm, *B. cereus* 17.5mm) and fruits (FAcE) (*P. aeruginosa* 17mm, *S. aureus* 15mm, *B. cereus* 17mm), while the limited effect of the aerial part methanol extract (AME) was on *S. aureus* 8mm and *B. cereus* 8mm. However, the aerial part aqueous extract (AAE) had an impact on the *S. aureus* (22mm) and *P. aeruginosa* (11.6mm), while the impact of the fruit aqueous extract (FAE) is limited on the *S. aureus* only (16.3mm). Weaker effect was observed with the tuber's extracts. Except tuber methanol (TME) and acetone extracts (TAcE) have an impact only

on the *B. cereus* (12.5 mm and 9 mm respectively). The results revealed that all extracts tested were effective against the microorganisms studied, except *E. coli* ATCC 25922. Gentamicin exerted highest inhibitory effect against the used strains than all plant extracts tested, while no inhibitory effect could be observed for DMSO, used as negative control.

The minimum inhibitory concentration (MIC) was determined for the extracts which showed the best antibacterial activity against the tested strains except *E. coli* ATCC 25922 that was resistant to all extracts used.

**TABLE 4: IN VITRO ANTI-BACTERIAL ACTIVITY OF A. LONGA EXTRACTS EVALUATED BY AGAR DISC DIFFUSION METHOD**

Plant material	Extract	Diameter of inhibition zone (mm)															
		<i>Escherichia coli</i> ATCC 2592 2				<i>Pseudomonas aeruginosa</i> ATCC 27853				<i>Staphylococcus aureus</i> ATCC 25923				<i>Bacillus cereus</i> ATTC 10876			
		Concentration (mg/mL)															
		200	100	50	25	200	100	50	25	200	100	50	25	200	100	50	25
Aerial parts (Stem and leaves) (AAE)	Water	-	-	-	-	11.6	8	-	-	22	18	15	-	7	-	-	-
Fruits (FAE)		-	-	-	-	-	-	-	-	16.3	14	8	-	8	-	-	-
Tubers (TAE)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aerial parts (Stem and leaves) (AME)	Methanol	-	-	-	-	-	-	-	-	8	-	-	-	8	-	-	-
Fruits(FME)		-	-	-	-	20	15	13	-	18.5	17	12	-	15	14	13	-
Tubers(TME)		-	-	-	-	-	-	-	-	-	-	-	-	12.5	9	7	-
Aerial parts (Stem and leaves)(AAcE)	Acetone	-	-	-	-	19	17	13	-	14	11	9	-	17.5	15	12	-
Fruits(FAcE)		-	-	-	-	17	11	9	-	15	11	9	-	17	14.5	12	-
Tubers(TAcE)		-	-	-	-	-	-	-	-	-	-	-	-	9	7	-	-
Gentamicin		-	-	35	-	-	-	25	-	-	-	30	-	-	24	-	-
DMSO		-	-	+	-	-	-	+	-	-	-	+	-	-	+	-	-

Diameters of inhibition round the disc are expressed in mm- : no inhibition+ : growth of bacteria

The data obtained from microdilution method for determining MIC is presented in (Table 5). Minimum inhibitory concentration (MIC) of the aerial aqueous extract (AAE) was 50 mg/mL for *P. aeruginosa*, 12.5 mg/mL for *S. aureus*, whereas the fruit aqueous extract (FAE) was 50 mg/mL for *S. aureus*. The aerial (AAcE) and fruit acetone (FAcE) extracts were effective against *P. aeruginosa*, *S. aureus* and *Bacillus cereus* and showed the same MIC, 12.5 mg/mL. A strong antibacterial activity is presented by a very low MIC<sup>44</sup>. The fruit methanolic extract (FME) gave the lowest minimal inhibitory concentration against *P. aeruginosa* and *S. aureus* with MIC values of 3.125 mg/mL, and 6.25mg/l for *Bacillus cereus*.

It is obvious that the antimicrobial activity of all extracts depends largely upon the concentration of extracts, the bacterial strains and the type of plant extract.

The extracts obtained were able to inhibit the growth of one or more of the tested standard strains to a certain percentage. The highest activity was shown in fruit methanol extract against *P. aeruginosa*, and *S. aureus* with a MIC= 3.125 mg/L, and 6.25 mg/L for *Bacillus cereus*. Further, *P. aeruginosa* is recognized as a dangerous pathogen owing to its resistance to many antibiotics and its capacity to acquire further resistance against progressively newly introduced antimicrobial agents. This organism is reported to be responsible

for a local nosocomial infection <sup>45</sup>, and for *S. aureus*. These results are very interesting since this microorganism can be commonly involved in skin infections <sup>23</sup>, while *Bacillus cereus* and *Bacillus*

*subtilis* have been known to act as primary invaders or secondary infectious agents in a number of diseases and have been implicated in some cases of food poisoning <sup>46</sup>.

**TABLE 5: DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (mg/ml) OF *A. LONGA* EXTRACTS EXPRESSED AGAINST BACTERIAL STRAINS**

Plant material	Extract	MIC (mg/mL)		
		<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Staphylococcus aureus</i> ATCC 25923	<i>Bacillus cereus</i> ATTC10876
Aerial parts (Stem and leaves) (AAE)	Water	50	125	
Fruits (FAE)			50	
Fruits (FME)	Methanol	3,125	3,125	6,25
Aerial parts (Stem and leaves) AAcE)	Acetone	12,5	12,5	12,5
Fruit (FAcE)		12,5	12,5	12,5

In previous studies <sup>23</sup>, Campores et al. showed an interesting antibacterial activity of dried extracts of *Aristolochia trilobata* against a few Gram-positive and Gram-negative bacteria. In another study, *Aristolochia paucinervis* Pomel, was reported as good antimicrobial drug <sup>47</sup>. Furthermore, Angalaparameswari et al., reported that ethyl acetate and methanol extracts of *Aristolochia bracteata* were found to be good antimicrobial agents <sup>48</sup>. However, plants belonging to the genus *Aristolochia* were reported to contain aristolochic acids <sup>1, 3</sup>. These compounds were shown to possess immunostimulatory and antiinflammatory properties <sup>43</sup>.

In fact, this compound isolated from *Aristolochia longa* was reported to be active against several aerobic bacteria <sup>49</sup>. A similar compound isolated from ethyl acetate and methanol extracts of *Aristolochia bracteata* showed good antimicrobial activity against both gram positive and gram negative bacteria <sup>48</sup>.

According to Balick and Arvigo, (1998) aristolochic acids were present in the methanol extracts and probably also in the water preparations of aerial parts of the plants used in the folk medicine <sup>50</sup>.

It is possible that these compounds could be responsible for the antibacterial activities reported here.

On the other hand, we note that the antibacterial activity of all extracts depends largely upon the

concentration and type of plant extracts, the solvent used and the bacterial strains.

The variability of the activity observed between these extracts may be due to the presence and concentration of bioactive compounds such as flavonoids, alkaloids, saponins and tannins. It is reported that flavonoids, phenolic compounds, tannins and alkaloids are the most important antimicrobial agent and bioactive constituents in plants <sup>51</sup>.

In the present study, we noticed that extracts obtained with acetone as solvent (aerial and fruit extracts) have a broad spectrum of activities compared to the methanol extract (only fruit extract) (**Table 1**) and this result may be due to the type of phenolics and their relative distribution but not only for their content. Polyphenol structure affects the bacteria tolerance to polyphenols <sup>52</sup>, moreover phytochemical investigation of acetone extract from *A. elengans* revealed the presence of lignanes, kaurane diterpenes and the sesquiterpene nerolidol and caparrapidiol <sup>53</sup>.

Acetone extract have been shown to possess the highest activity when compared to other solvents <sup>38, 30</sup>. This activity was attributed to the volatile oil rich with constituents and bearing different functionalities. In our study, we support the fact that methanol and acetone are the best solvents. This applies to other studies indicating that methanol, methanol–water, and acetone were the best solvents in extracting antimicrobials and

antioxidants (i.e., polar constituents such as phenolics) from plant materials<sup>54,55</sup>.

Tuning the polarity of the organic solvents and extraction conditions could yield components with selectivity and high antimicrobial effects<sup>30</sup>.

The aqueous extract shows less inhibition zone than the other extracts or none inhibition of the bacterial strains (tuber extract) at all the concentrations tested in comparison to other extracts. This is in consonance with the results of a study reporting water to be less effective than organic solvents at extracting the active compounds from plants<sup>56</sup>, and other studies show that though water has maximum polarity among the chosen solvents, the water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are only important as antioxidant compounds<sup>57</sup>.

The lack of antibacterial activity in some of the concentrations of the extract is not surprising as a number of plant extracts which have been found ineffective against certain test organisms at lower concentrations and may be attributed to the presence of lesser amounts of the antimicrobial compounds<sup>58</sup>. But in our study, the results revealed that *E.coli* ATCC 25922 was resistant to all extracts used. In previous studies, it has been reported that this bacteria was not inhibited by the chloroform extract of *Aristolochia trilobota* leaves and bark<sup>23</sup>, similarly<sup>47</sup> none of the fractions of *Aristolochia paucinervis* Pomel showed any activity against *E. coli* ATCC 25922 up to 1 mg/mL. The same result was shown in other studies where *E.coli* ATCC 25922 was resistant to almost extracts tested<sup>30,31</sup>.

The antibacterial effects of the extracts could be explained by disturbance of the permeability barrier of the bacterial membrane structure<sup>59</sup>.

In fact, active principles singly or in combination inhibit greatly the life processes of microbes, by binding with their protein molecules, acting as chelating agents, altering their biochemical systems, preventing utilization of available nutrients to the microorganisms<sup>60</sup>. The antibacterial effect of phenolic compounds might be related to the interaction with enzymes, adsorption to cell membranes, substrate and metal ion deprivation<sup>61</sup>.

Our study is in accordance with previous studies which indicate that the antibacterial activities depend considerably on extraction method, the solvent nature and the strain tested<sup>62,58,31</sup>.

**Antifungal activity:** *A. longa* L. extracts obtained in this study were unable to inhibit the growth of the fungi tested (*Aspergillus flavus* NRRL 391 and *Aspergillus niger* 2CA 936), and yeast (*Candida albicans* ATCC1024).

**CONCLUSION:** In summary, the present study showed that the biologically active constituents from *A. longa* L. can be obtained by different extraction solvents. The extraction methods as well as the part of the plant used had a big influence on the antioxidant, antibacterial and anti-inflammatory properties of extracts.

These results could justify the use of this plant in traditional pharmacopeia for the treatment of certain diseases; however, it is interesting to characterize the compounds responsible of this biological activity.

**ACKNOWLEDGMENTS:** We would like to thank Algerian Ministry of High Education and Research for the financial support as well as the Applied Microbiology Laboratory, University F.A.Setif1 for technical assistance.

**CONFLICT OF INTEREST STATEMENT:** We declare that we have no conflict of interest.

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**How to cite this article:**

Merouani N, Belhattab R and Sahli F: Evaluation of the biological activity of *Aristolochia longa* L. Extracts. Int J Pharm Sci Res 2017; 8(5): 1978-92. doi: 10.13040/IJPSR.0975-8232.8(5).1978-92.

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