CONTRIBUTION TO THE STUDY OF LEAVES : *AZADIRACHTA INDICA* A. Juss (MELIACEAE) : EVALUATION OF THE ANTIOXIDANT ACTIVITY

ABSTRACT

In Africa, the use of plants for therapeutic purposes is an ancient practice. In recent years, much scientific work has been spent to the chemistry and toxicology of medicinal plants; there is a particular focus on natural antioxidants in relation to their various therapeutic properties. Therefore, the purpose of our study is to determine the antioxidant activity of aqueous and hydroethanol extracts from the leaves of *Azadirachta indica* A.Juss a plant known for its many pharmacological properties.

To do this, we worked on leaves of *Azadirachta indica* A. **Juss** dried in the oven at 60 degrees for 24 hours and reduced to fine powder. Then we extracted first with distilled water to obtain the water extract and then with a mixture of distilled water and ethanol at 50/50 (v/v) to obtain the hydro-ethanol extract. Antioxidant activity was done through the DPPH test, the FRAP method and the fixation of the radical nitro-oxide (NO).

The results revealed that for the DPPH test, the hydro-ethanol extract is more active (IC_{50}=9.9±0.14 mcg/ml) compared to the activity of the water extract (IC_{50}= 11±0.28 mcg/ml). For the FRAP method, we note absorbance of 0.56 and 1.05 respectively for water and hydro-ethanol extract at a concentration (166.7 µg/ml). On the other hand, for the inhibition of radical nitro-oxide (NO), activity is low for the two extracts of *Azadirachta indica* A. Juss respectively of 36.94±2.1% for the aqueous extract and 26.03±2.52% for the hydroethanol extract.

The results of this work highlight the antioxidant properties of *Azadirachta indica* A. Juss leaves extracts. These results give credit to certain ethnopharmacological uses of *Azadirachta indica* A. Juss, but, study benefits must be carried out to support this use especially on toxicology.

Keywords: Leaves, *Azadirachta indica* A. Juss, antioxidant activity, DPPH, FRAP, NO.

IC_{50}: 50% inhibitory concentration. DPPH: 2, 2 Diphenyl-1-picrylhydrazil. FRAP : Ferric Reducing Antioxidant Power. NO : oxide nitric radical

1. INTRODUCTION

The use of synthetic antioxidant molecules is currently being questioned because of the potential toxicological risks. Now, new plant sources of natural antioxidants are being searched **[12].**

Indeed, polyphenols are natural compounds that are widespread in the plant kingdom and that are of growing importance, in particular because of their beneficial effects on health **[4].** Their role as natural antioxidants is attracting more and more interest in the prevention and

treatment of cancer, inflammatory and cardiovascular diseases. In addition, they are also used as additives in the agri-food, pharmaceutical and cosmetic industries **[14].**
Scientific research has been developed for the extraction, identification and quantification of these compounds from different sources such as agricultural and horticultural culture or medicinal plants **[5].**
This approach will significantly increase the number of plant-derived discoveries of natural antioxidants, which could help solve the growing problem of the carcinogenicity of currently available synthetic food additives and also combat diseases in which stress oxidative is involved. It is with this in mind that our subject, which is about on the research of the antioxidant activity of the leaves of a plant of the West African pharmacopoeia, Azadirachta indica A. Juss by three methods (DPPH, FRAP and NO).

2. MATERIALS AND METHODS

2.1 Study Area, Collection and Identification of Plant Materials

This work was carried out at the Special Research Center Department of Pharmacognosy and Botany, Cheikh Anta Diop University, Dakar, Sénégal.

The plant sample was collected from in this Cheikh Anta Diop University. The plant specimen was identified by a specialist of botany in the laboratory of Botany, Cheikh Anta Diop University, Dakar, Sénégal.

2.2 Preparation of Plant Sample

The leaves were then oven dried at 60°C for few days and was crushed into powders in a mechanic grinder.

2.3 Sample extraction

The extraction was carried out by decoction of 100 g of leaf powder, boiled under reflux in 400 ml of water for 30 minutes. After filtration, the aqueous extract thus obtained was evaporated with Rotavapor to obtain a dry residue. Finally 50 g of the leaf powder is extracted successively with 400 ml of water and 400 ml of ethanol by decoction several times. The decoction obtained is concentrated in a rotary evaporator and stored on a watch glass and then put in an oven to be dried (60 ° C).

2.4 Methods

The determination of trapping capacities was done using three methods: DPPH, FRAP and NO.

- **DPPH**

The antioxidant capacity was evaluated according to the method described by Molyneux in 2003 **[6].** The extract was tested at different concentrations (1.56-3.125-6.25-12.5-25-50-100 and 200 mcg / ml) with DDPH in the following volumes of volumes extracted / DDP (1/4). Ascorbic acid was used as a reference antioxidant and tested at the same concentrations. Absorbance measurement was performed at 517 nm spectrophotometer after 30 minutes incubation (T30) using ethanol as a blank. Three tests were performed for each test portion

concentration (n = 3). The results are first expressed in percentage inhibition (PI equal to the absorbance of the DPPH alone minus the absorbance after adding the extract to a given concentration divided by the DPPH absorbance alone) of the anti-radical activity and IC_{50} (Concentration in free radical to trap 50% free radicals). Then, the EC_{50} calculated from the IC_{50} divided by the molar mass of the DPPH and anti-radical power (PA) equal to the inverse of the effective concentration **[1].**.

- **FRAP**

The reducing ability of leaf extracts was assessed using the method set by Bassene, 2012. Briefly, various concentrations of each extract (2,6-5,2-10,4-20,8-41,7-83,3 and 166.7 mcg / ml) were diluted to half in distilled water and then mixed with 2.5 ml of phosphate buffer (0.2M; pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] at 1%. The mixtures obtained are incubated at 50 ° C. for 30 min. after, 2.5 ml of trichloroacetic acid (10%) is added. After centrifugation at 3000 rpm for 10 min, 2.5 ml of the supernatant of each concentration is mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The absorbance is measured at 700 nm using a spectrophotometer (BTS 350, biosystems).

- **NO**

Add 1 ml of sodium nitroprusside and 250 µl of distilled water in clean and dry tubes, mix all then add 250 µl of each dilution (1/2) extracts the concentrations 166.67 and 333.3 (mcg / ml), mix and incubate for 150 min on the laboratory bench.After incubation, 500 µl of each tube prepared above are taken and introduced into a new clean, dry tubes and 1 ml of sulfanilic acid solution are added. The mixture is homogenized and allowed to incubation for 5 to 10 min. Naphthylethylènediamine 1 ml is added to each tube and the whole is homogenized slowly and incubated again for 30 min. The absorbance of each tube is read at 540 nm.Inhibition percentages are calculated from the following formula:

$$\%I_{NO} = \left(\frac{A_t - (A_E - A_B)}{A_t}\right)$$

INO% is the percentage of inhibition of the nitro-oxide radical, At is the absorbance of the negative control (500 ml of distilled water and 1 ml nitroprusside), AE is the absorbance of the test sample in the presence of , AB is the absorbance of the blank (500 ml of distilled water, 1 ml of sulfanilic acid and 1 ml of ethylene naphthyl).

- **Statitiscal analysis**

Statview software was used for statistical analysis. Thus, a normal analysis of variance followed by the Fischer test was performed. The difference is considered significant if $p < 0.05$ compared to the negative control (DPPH solution). Statgraphics 5.0 software was used to generate inhibitory concentrations.Variances analysis was performed using the Fisher test at a significance level of 0.05 using Statview software.

3. RESULTS

3.1 DPPH

The results of the determination of the antioxidant activity of the extracts by the DPPH method expressed as percentage of inhibition are shown in figure 1.

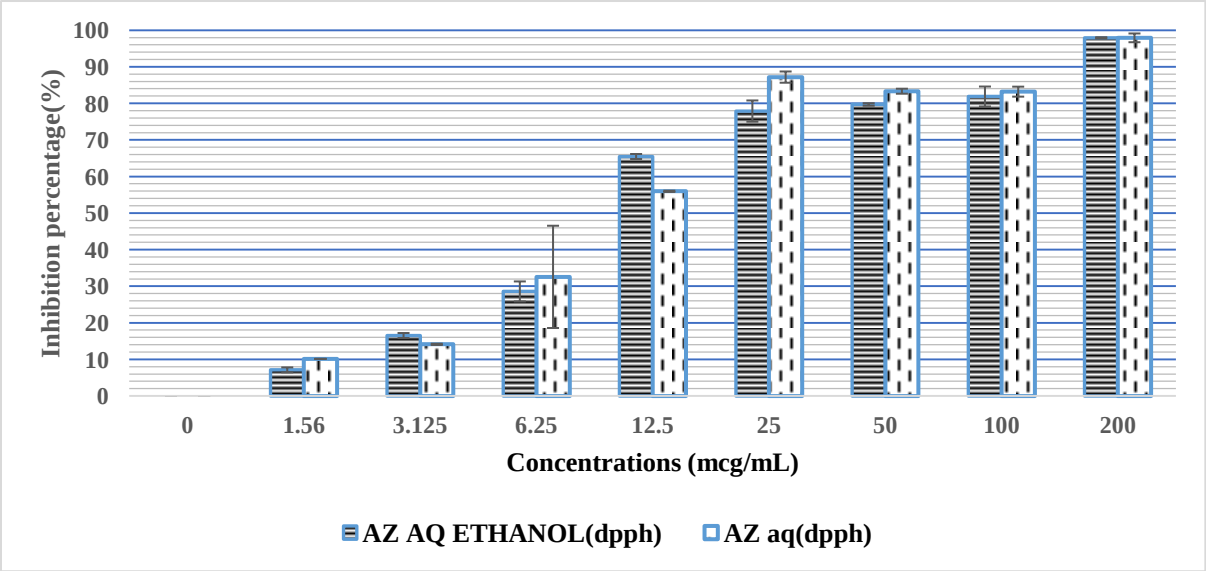

Fig. 1. Evolution of DPPH reduction as a function of the concentrations of each extract tested

Azaq (dpph) = aqueous extract of *Azadirachta indica* on DPPH; AZ ETHANOL (dpph) = ethanolic extract of *Azadirachta indica* on DPPH

The histogram of Figure 2 gives so compared the different IC_{50} extracts, gallic acid and tannic acid. The latter two compounds were used as positive controls.

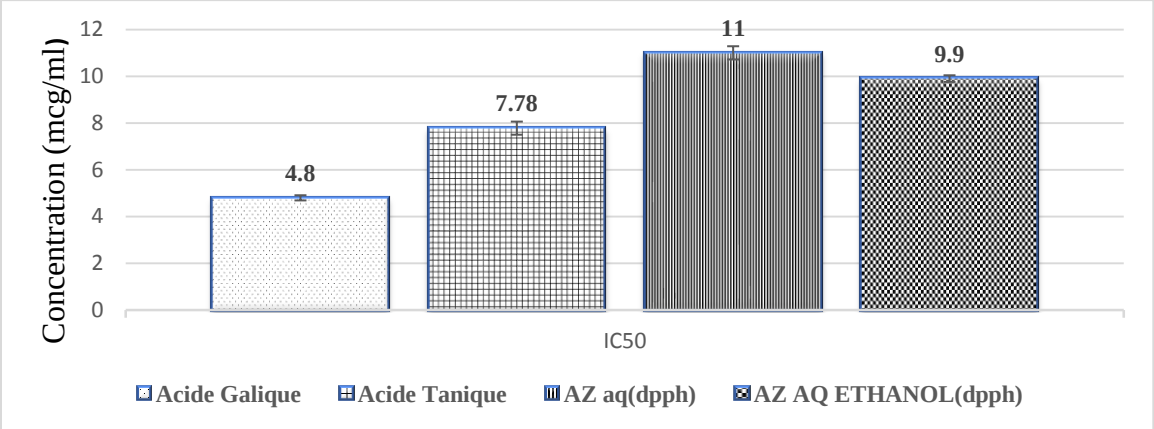

Fig. 2. Histogram of inhibitory concentrations 50 different extracts tested and positive controls

Azaq (dpph) = aqueous extract of *Azadirachta indica* on DPPH; AZ ETHANOL (dpph) = ethanolic extract of *Azadirachta indica* on DPPH

3.2 FRAP

The results of determining the reducing power of the extracts by the FRAP method expressed as reducing power are shown in figure 3.

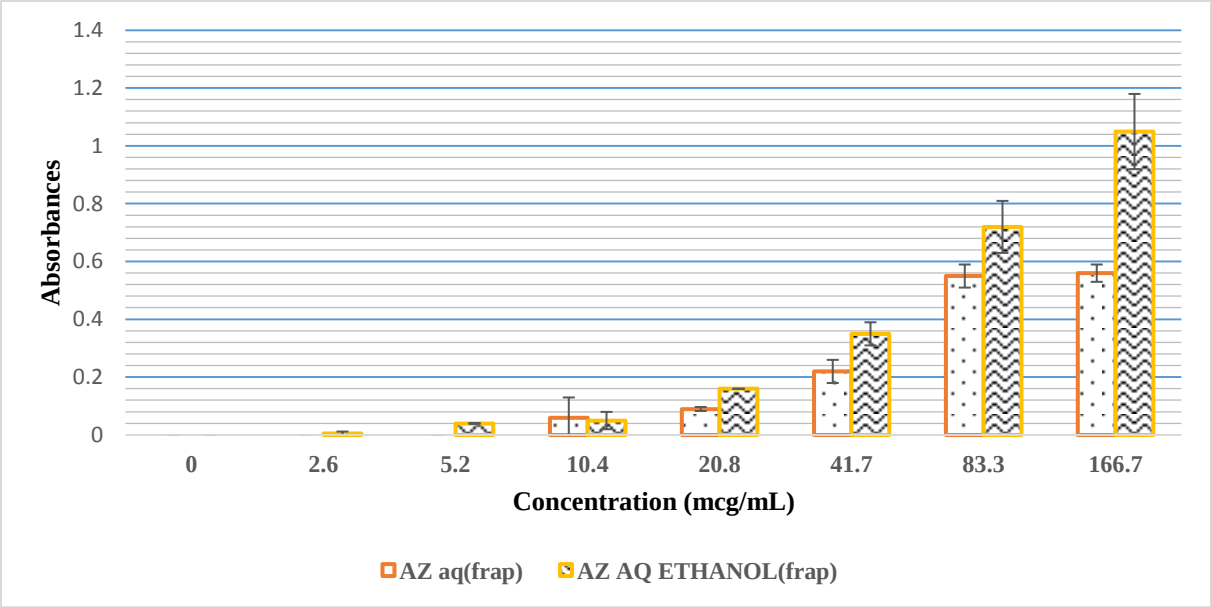

Fig. 3. Evolution of the reduction of Fe^{3+} to Fe^{2+} according to the concentrations of each plant extract

Azaq (frap): aqueous extract of *Azadirachta indica* Azaq ETHANOL (frap): hydro-ethanol extract of *Azadirachta indica*.

We used gallic acid as a standard but at concentrations much lower than those of our extracts. It is for this reason that one can not superimpose the histograms for a better simile of our extracts with our standard. The latter is much more active than our extracts. Here represented the histogram of our standard in Figure 4.

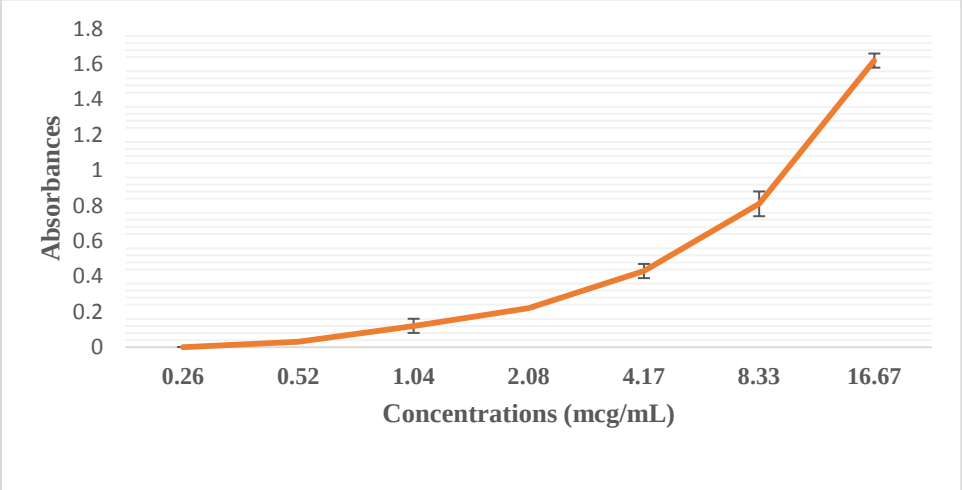

Fig. 4. Evolution of the reduction of Fe^{3+} to Fe^{2+} gallic acid

3.3 NO

The results of the determination extracts of inhibition percentages by the NO method are shown in Figure 5.

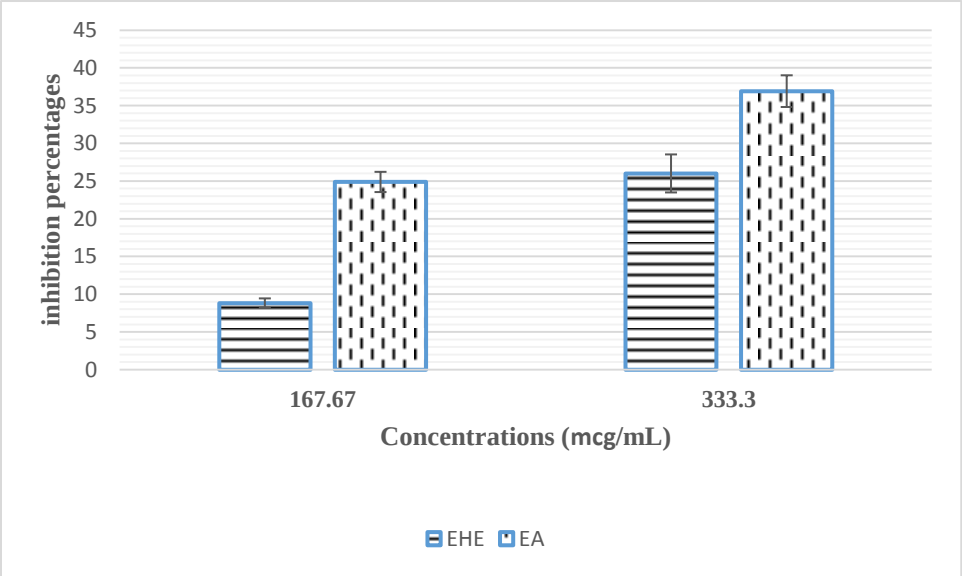

Fig. 5. Percentage inhibition of extracts by the NO method

EHE: Hydro-ethanolic extract; EA: Aqueous extract

Tannic acid was used as a control but at much lower concentrations than our extracts. Figure 6 below shows the results of the control by the NO method.

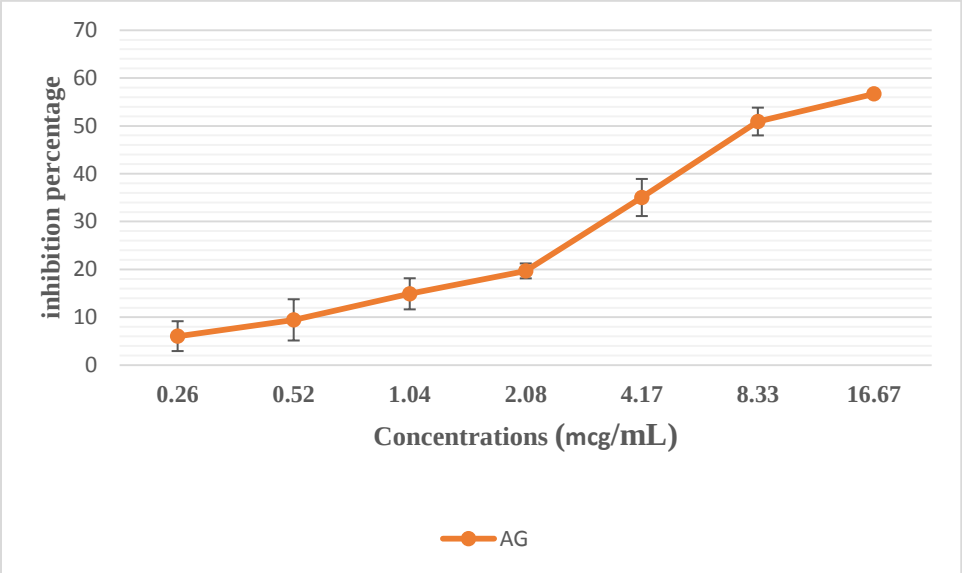

Fig. 6. Percentage inhibition of NO fixing by tannic acid

4. DISCUSSION

Our study aimed to research the antioxidant activity on the leaves of *Azadirachta indica* A.Juss by using three simple methods and practices.To achieve this aim we worked on two

extracts: aqueous and hydro-ethanolic extracts of the leaves of *Azadirachta indica*.For extracts, we performed two extractions decoction: one with distilled water and the other with a mixture of water and ethanol. The choice of solvents is due to the fact that they are polar and able to extract compounds such as alkaloids, tannins and flavonoids (polyphenols) **[1]** found in the leaves of Azadirachta indica and mime better extraction made in the traditional practice for preparing extracts.

To evaluate the antioxidant properties of our two extracts, we used several tests, including the DPPH method, the FRAP method and the fixing of the nitro-oxide radical.
The method at $DPPH^{\cdot}$ is a simple but highly effective method **[7].** The results of the anti-radical activity on the radical DPPH. , show that at all concentrations tested, the two extracts have antioxidant activity and they are able to trapping the radical $DPPH^{\cdot}$. At the concentration of 100 mcg / ml, the percentage inhibition is 83.23 ± 1.19% for the aqueous extract and 81.91 ± 2.73% for the hydroethanolic extract. The highest activity is observed at the concentration of 200 mcg / ml with a percentage inhibition of 97.95 ± 0.17% for both extracts. The work of Nahak et al **[8].**on the methanolic extract of *Azadirachta indica* leaves reveals a percentage inhibition of 41.17 ± 0.04% at the concentration of 100 mcg / ml. This difference in activity could be explained by the nature of the solvent used but also by intrinsic or extrinsic factors related to the plant.

The calculation of IC_{50} allows us to compare the antioxidant activity of aqueous extracts, hydro-ethanolic leaves and also those references. The lower the IC_{50}, the higher the antioxidant activity of the compound.The IC_{50} of the hydro-ethanolic extract (IC_{50} = 9.9 ± 0.14 mcg / ml) is low compared to that of the aqueous extract (IC_{50} = 11 ± 0.28 mcg / ml) which indicates a better activity of the latter; this could be explained by the richness of the hydro-ethanolic extract in compounds having a labile hydrogen such as polyphenols **[2].** This is in agreement with the work of Pandey et al. **[10]** on the ethanolic extract of leaves of *Azadirachta indica* which showed that this extract had an anti-radical activity on the $DPPH^{\cdot}$ and that this activity was similar to the content of the extract in polyphenolic compounds.

However, the IC 50's of gallic acid (IC_{50} = 4.8 ± 0.11 mcg / ml) and tannic acid (IC_{50} = 7.78 ± 0.28 mcg / ml) used here as a reference remain low relative to at IC_{50} extracts.
The use of the FRAP test confirms the antioxidant activity found with the DPPH method; both tests having different principles. The results show that the two extracts of *Azadirachta indica* have a Fe^{3+} reducing activity at Fe^{2+} at the concentrations tested. At these concentrations (10.4 mcg / ml, 20.8 mcg / ml, 41.7 mcg / ml, 83.3 mcg / ml, 166.7 mcg / ml), the observed absorbances are (0.06 0.09, 0.22, 0.55, 0.56) for the aqueous extract and (0.04, 0.16, 0.35, 0.72, 1.05) for the hydro -éthanolic extract. The reduction of Fe^{3+} in Fe^{2+} changes with the growth of the concentration of our extracts. The gallic acid used as reference has an absorbance of 1.61 for a concentration of 16.67 mcg / ml and therefore has a higher activity than that of the extracts.

The reducing activity of the extracts could be explained by the presence of compounds that would yield while remaining stable electrons. Therefore, antioxidants are considered reducing and oxidative inactivators **[12].** The work of Olabinri et al **[9].**on the aqueous extract of *Azadirachta indica* has shown that this extract has a reducing activity; after determination of total polyphenols of the extract, the authors concluded that the activity was due to the flavonoid content and polyphenols contained in the extract. Some previous studies also explain that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity **[3].**

Fixing the NO radical remains low for both extracts as inhibition percentages do not reach 50%. In our literature searches, we found no scientific publications have studied the fixing of NO radical by leaf extracts of *Azadirachta indica*. Gallic acid and tannic acid used as a reference respectively have IC_{50} values of 9.16 ± 1.2 mcg / ml for tannic acid and 8.74 ± 1.6 mcg / ml for gallic acid. The antioxidant activity present in both extracts of *Azadirachta indica* could be related to its chemical composition including flavonoids, polyphenols and tannins **[2].**

Indeed, the leaves of Azadirachta indica are characterized by the presence of alkaloids, quercetin, β-sitosterol, flavonoids, saponins, tannins, vitamin C and carotene **[10, 11]**. In general, polyphenols are known for their antioxidant power. Flavonoids act mainly as antioxidants, by stabilizing peroxide radicals or by deactivating oxygen species: superoxide anion, hydroxyl radical, singlet oxygen.

Polyphenols are an important family of antioxidants found in plants. They are excellent scavengers of ROS and great transition metal chelators such as iron and copper **[13].**Thus, whatever the nature of the anti-radical power of our extracts, it should be noted that there is a correlation between phenolic content and antioxidant activity of our extracts. What joint some authors in their conclusions including **[2, 10]**.

5. CONCLUSION

To ending we can say that leaves *Azadirachta indica* A. Juss have a very important antioxidant power. This is even confirmed by its use of populations to treat ailments such as malaria. The results confirm that these parts of the plant can be used as having anti-inflammatory properties antibacterial, antiviral, antioxidant and immunostimulant. Also, with the high cost of synthetic origin antioxidant products, the use of more accessible sheets could be an alternative for the equilibration of the pro-oxidant balance / antioxidant among the poor. Finally, studies in thought could lead to isolate and identify antioxidant molecules with a guided bio method but also to determine the acute toxicity and subacute fruits and stalks.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1- **Bassene E.** Initiation à la recherché sur les substances naturelles : Extraction – Analyses – Essais biologiques. Presses universitaires de Dakar, **2012,** 150 pp.

2- **Bharat P, Rijal S, Raut S, And Pandeya A.** Investigations of antioxidant and antibacterial activity of leaf extracts of Azadirachta indica. African Journal Biotechnology, Vol 14(46), **2015.**pp 3159-3163.

3- **Jeong S.M., Kim S.Y., Kim D.R., Jo S.C., Nam K.C., Ahn D.U., et Lee S.C.** Effects of heat treatment on the antioxidant activity of extracts from citrus peels. Journal of Agriculture and Food Chemistry. **2004.**52, pp3389–3393.

4- **Koechlin-Ramonatxo C.** Oxygen, oxidative stress and antioxidant supplementation, or another way for nutrition in respiratory diseases. Nutrition Clinique et Métabolique. **2006,** 20, 165- 177.

5- **Marc Fr., Davin A., Deglène-Benbrahim L., et Ferrand C.** Méthodes d'évaluation du potentiel antioxydant dans les aliments. Erudit, M/S: médecine sciences. 20(4), **2004**.pp458-463

6- **Molyneux P.** The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Techno. **2003.**26: 211-219.

7- **Moon J-K And Shibamoto T.** Antioxidant Assays for Plant and Food Components. J. Agric. Food Chem., 57 (5), **2009.**pp.1655-1666.
8- **Nahak G, Sahu R.K** In vitro antioxidative activity of *Azadirachta indica* and *Melia azedarach* Leaves by DPPH scavenging assay. Nature and Science, **2010.**vol 8(4),pp22-28.
9- **Olabinri B M, Adebisi, J A. Odesomi, O F Olabinri P F And Adeleke G E.** Experimental classification of the antioxidant capacity of the leaf, stem and root barks of *Magnifera indica* and *Azadirachta indica*. African Journal of Biotechnology, **2009.**vol 8 (13), pp2968-2972.
10- **Pandey G, Verma Kk, Singh M.** Phytochemical, Antibacterial and free radical scavenging properties *of Azadirachta indica* (Neem) leaves. Int J Pharm Sci, **2014.**Vol 6, Issue 2, 444-447.
11- **Pharmacopée D'Afrique De L'Ouest (Pao).** Organisation Ouest Africaine de la Santé, CEDEAO ECOWAS. **2013.** Pp 44-46, 253p
12- **Siddhuraju P.et Becker K.** The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata (L.*) Walp.) seed extracts. Food Chemistry. **2007.**101(1), 10-19.
13- **Strack D.** Phenolic metabolism. In Plant Biochemistry, Dey PM, Harborne JB Eds, Academic Press, San Diego, pp. 387-416. Thompson M, Williams CR (1976). Stability of flavonoids complexes of copper (II) and flavonoid antioxidant activity. Anal. Chem. Acta. **1997.** 85: 375-381
14- **Vârban D.I., Duda M., Vârban R., et Muntean S.** Research Concerning the Organic Technology for *Satureja Hortensis L*. Culture. Bulletin UASVM Agriculture. **2009,** 66(2), 225- 229.