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Evaluation of the anti-radical activity of methanolic and aqueous extracts of stem, stem bark and leaves of *Waltheria indica* by scavenging the free radical cation ABTS

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Abstract

Background: *Waltheria indica* is an antioxidant rich flowering plant species in its various parts and usually used in sub-Saharan Africa's traditional medicine. The aim of this work was to evaluate the anti-radical activity of different concentrations of aqueous and methanolic extracts of stem, stem bark and leaves of *Waltheria indica*, in order to determine the most antioxidant organ of the plant on the one hand and the type of extraction allowing to collect the maximum of bioactive compound on the other hand.

Methods: The method used is based on the measurement of the free radicals of the radical cation of 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS^{•+}) compared to that of a reference antioxidant, the gallic acid.

Results and Discussion: With the methanolic extraction, the results obtained showed that the stem bark was the most active organ followed by the stem and then the leaves. The IC₅₀ obtained were: for the stem bark (IC₅₀ = 4.3 µg.mL⁻¹); for the stem (IC₅₀ = 6 µg.mL⁻¹); and the leaf (IC₅₀ = 37.5 µg.mL⁻¹). Gallic acid, the referenced antioxidant, showed an IC₅₀ of 0.41 µg.mL⁻¹. Regarding the extraction method, aqueous extraction had a higher yield (IC₅₀ of stem aqueous extract = 2.5 µg.mL⁻¹) compare to methanolic extraction (IC₅₀ of stem methanolic extract = 6 µg.mL⁻¹).

Conclusion: Aqueous extraction showed a better yield than methanolic extraction. The various organs of *Waltheria indica*, particularly the stem bark, can provide natural antioxidants that can be used in preventive medicine.

Keywords: *Waltheria indica*, aqueous extracts, methanolic extracts, gallic acid, anti-radical activity

1. Introduction

Antioxidants are compounds that protect the human body from damage caused by free radicals, hence their great interest in the scientific community. In fact, in humans, the oxidative damage to biological molecules (DNA, lipids and proteins) is associated with cardiovascular diseases [1], certain cancers [2], diabetes [3], Alzheimer's disease [4] as well as the aging process [5]. Antioxidants are also of great interest in the food and cosmetics industry because they delay rancidity and ensure the maintenance of the nutritional qualities of foods [6-8]. The potential harmfulness of synthetic antioxidants is at the root of the renewed interest in antioxidants naturally present in natural products such as plants. Indeed, a very wide variety of natural antioxidants are phenolic compounds mostly extracted from plants [9]. These inexpensive compounds, potentially less toxic than synthetic antioxidants, could protect the human body and food in which they are added as extracts from oxidative damage. However, the composition and the amount of bioactive substances in a plant being variable from one part to another, the antioxidant activity of the plant can therefore vary depending on the organ [10]. In addition, the biological activity of plants will depend on the method of extraction of its bioactive substances [11].

Waltheria indica (*W. indica*) is a flowering plant species rich in bioactive compounds such as caffeic acid, flavonoids, alkaloids, sugars and tannins giving it a powerful therapeutic effect [12]. *W. indica* it is a medicinal plant commonly known as sleepy morning, is distributed widely in tropical regions of the world [12].

Based on the ethic approach, evidences on the pharmacological properties of *W. indica* are available [12]. Indeed, different parts of *W. indica* are used in traditional medicine in several tropical regions of the world and in particular in sub-Saharan Africa. Thus, infusions from all parts of the plant are used in the treatment of coughs, as eye baths, in the treatment of ulcers, lung infections, in the treatment of skin diseases, in the treatment of diarrhea, rheumatism, epilepsy, sexual impotence due to its aphrodisiac properties, typhoid fever, malaria, fatigue, treatment of asthma in adults etc. [12]. Also, according to the literature, different types of extracts from different parts of *W. indica* have shown a large number of therapeutic and pharmacological properties on animal models such as: sedative [13], analgesic [14], hematinic [15], aphrodisiac [16], anti-inflammatory [17], anti-microbial activities [18], anti-cataract [19], anti-diabetic [20], anti-cancer [21] and antioxidant [20] due to the presence of phenolic compounds which scavenge free radicals such as flavonoids and tannins [22]. Indeed, according to the literature, the methanolic extracts of the roots of *W. indica* and of the whole plant exhibit antioxidant activity by inhibiting 1,1-diphenyl-2-picrylhydrazyl (DDPH) [23]. The aqueous leaf extract inhibits lipid peroxidation in rats [14]. According to Garba *et al.* [24] The hexane extract of *W. indica* leaves has antioxidant activity equivalent to that of ascorbic acid but greater than that of tocopherol. An antioxidant activity of hydroalcoholic extract of the roots of *W. indica* has also been observed [25]. While it is well established that the entire *W. indica* plant, its leaves, stem and roots are composed to varying degrees of "flavonoids, tannins" antioxidant compounds [22]; a comparative study of the antioxidant activity of different parts of *W. indica*, however, is not reported in the literature. Also, in this study, we set out to evaluate the anti-free radical activity of aqueous and methanolic extracts of various organs of *W. indica* from Gabonese soil. This is to determine on the one hand which part of the plant is the most active and on the other hand which method of extracting these bioactive compounds provides the best yield. The anti-free radical activity is measured by scavenging the radical cation of 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS^{•+}) according to the method developed by Re *et al.* [26] and optimized by N'negue *et al.* [27] with gallic acid as the reference antioxidant.



Image 1: *W. indica* (stem and leaves).

2. Materials et Methods

2.1. Materials

The plant material namely: the stem, stem bark and leaves

come from *W. indica* picked in the area named Okala in the North suburb of Libreville in Gabon. ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]), gallic acid, potassium persulfate (K₂S₂O₈) and hydrated sodium dihydrogen phosphate were purchased from Sigma-Adrilch (Saint-Quentin Fallavier, France). The water used was distilled by the equipment of the "Milli-Q Labo" laboratory (Millipore Japan, Tokyo, Japan). The anti-free radical activity was determined by UV spectrophotometry: V-200 spectrophotometer (BOECO, Germany). The optical density was read at 734 nm, the maximum absorption wavelength of the radical cation ABTS^{•+}.

2.2. Preparation of methanolic and aqueous extracts of *Waltheria indica*

The plant material is rinsed and then dried in a dryer, ventilated place, protected from light for two weeks. It is then ground into powder in a mill. The drug is extracted directly into water or methanol. The methanolic extracts of the stem, stem bark and leaves in tar form are diluted in DMSO.

2.3. Preparation of "reference antioxidant" gallic acid solutions

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is an aromatic organic compound, used as a reference anti-free radical compound. Ten working solutions, of decreasing concentrations, ranging from 0.94 to 0.094 μM, were prepared by diluting gallic acid in distilled water.

2.4. Measurement of anti-free radical activity

The principle of the test for measuring the anti-radical activity by the ABTS method is based on the decrease in the absorbance at 734 nm of the radical cation ABTS^{•+} (blue-green coloration) in the presence of a potentially anti-compound radical which reduces the radical cation. The reduction in the radical form of ABTS^{•+} causes discoloration of the solution.

The ABTS^{•+} radical ion is obtained by reacting the ABTS molecule (7 mM) with potassium persulfate (2.45 mM), in distilled water for 16 hours at room temperature and under cover light. The ABTS^{•+} solution obtained is diluted with sodium phosphate buffer (5 mM, pH = 7.4), in order to obtain a stock solution having an initial absorbance value at 734 nm between 0.65 and 0.70. The radical cation (ABTS^{•+}) is stable for more than 2 days when stored at room temperature and protected from light. All the assays were carried out three times and the anti-free radical activity is calculated according to the formula below.

$$\text{Anti-radical activity (\%)} = \left[1 - \frac{(Ar - Ab)}{(Ai - Ab)} \right] \times 100$$

(Ar, absorbance of the remaining ABTS^{•+} radical / Ai, absorbance of the initial ABTS^{•+} radical / Ab, absorbance of the phosphate buffer).

In fact, the reduction of the ABTS^{•+} radical cation therefore comes down to determining the anti-free radical activity and in total, the antioxidant properties of the extracts of *W. indica* compared to the antioxidant properties of gallic acid (standard). The anti-free radical activity was determined by UV spectrophotometry in cuvettes with an optical path of 1 cm (reaction volume of 2 mL). The incubation time was 6 minutes.

3. Results

3.1. Anti-free radical activity of gallic acid depending on the concentration

The anti-free radical activity increases linearly with the concentration of gallic acid (Fig. 1). An anti-free radical activity of 16% (± 3.5) was observed for a concentration of $0.094 \mu\text{g.mL}^{-1}$ of gallic acid. This activity reaches 59.45% (± 2.17) for a concentration of $0.47 \mu\text{g.mL}^{-1}$ and 100% for a concentration of $0.84 \mu\text{g.mL}^{-1}$ of gallic acid. The "benchmark antioxidant" gallic acid IC_{50} is $0.35 \mu\text{g.mL}^{-1}$.

3.2. Anti-free radical activity of aqueous and methanolic extracts of *W. indica* stem

3.2.1. Evaluation of the anti-free radical activity of the aqueous extract

According to the results obtained (Fig. 2), the anti-free radical activity of the aqueous extract of the stems increases with the concentration. It is $5.54 \pm 0.55\%$ for an aqueous rod extract concentration of $0.05 \mu\text{g.mL}^{-1}$; $8.5 \pm 0.02\%$ for a concentration of $0.5 \mu\text{g.mL}^{-1}$; from $45.17 \pm 2.24\%$ for a concentration of $1.25 \mu\text{g.mL}^{-1}$ from $57.14 \pm 0.54\%$ for a concentration of $5 \mu\text{g.mL}^{-1}$; $91.82 \pm 1.22\%$ for a concentration of $12.5 \mu\text{g.mL}^{-1}$ and 100% for a concentration of $25 \mu\text{g.mL}^{-1}$ and more in aqueous extract of the stems. The IC_{50} of the aqueous stem extract is $2.5 \mu\text{g.mL}^{-1}$.

3.2.2. Evaluation of the anti-free radical activity of the methanolic extract

The results (Fig. 3) show an anti-free radical activity which increases with the concentration of methanolic stem extract then stabilizes. In fact, the anti-free radical activity of the methanolic extract is only $1.58 \pm 0.28\%$ for a concentration of $0.05 \mu\text{g.mL}^{-1}$; $4.72 \pm 0.88\%$ for a concentration of $0.5 \mu\text{g.mL}^{-1}$; from $18.15 \pm 1.81\%$ for a concentration of $1.25 \mu\text{g.mL}^{-1}$ from $42.66 \pm 3.99\%$ for a concentration of $5 \mu\text{g.mL}^{-1}$; $86.41 \pm 1.08\%$ for a concentration of $12.5 \mu\text{g.mL}^{-1}$ and 100% for a concentration of methanolic extract of stems $\geq 25 \mu\text{g.mL}^{-1}$. The IC_{50} is $6 \mu\text{g.mL}^{-1}$.

3.3. Anti-free radical activity of methanolic extract of *W. indica* stem bark

According to the results obtained (Fig. 4), the anti-free radical activity increases with the concentration of methanolic extract of the stem bark. The anti-free radical activity of is $3.79 \pm 2.1\%$ for a concentration of $0.05 \mu\text{g.mL}^{-1}$; $6.75 \pm 2.39\%$ for a concentration of $0.5 \mu\text{g.mL}^{-1}$; from $21.73 \pm 1.75\%$ for a concentration of $1.25 \mu\text{g.mL}^{-1}$ from 59.79% for a concentration of $5 \mu\text{g.mL}^{-1}$; of $85.61 \pm 0.35\%$ for a concentration of $12.5 \mu\text{g.mL}^{-1}$. For a methanolic extract concentration of $25 \mu\text{g.mL}^{-1}$ and above, the anti-free radical activity is 100%. The IC_{50} for methanolic stem bark extract is $4.3 \mu\text{g.mL}^{-1}$.

3.4. Anti-free radical activity of methanolic extract of *W. indica* leaves

The results obtained (Fig. 5) show a linear increase in anti-free radical activity with the concentration of methanolic leaf extract. The anti-free radical activity of the extract is $1.94 \pm 1.30\%$ for a concentration of $0.05 \mu\text{g.mL}^{-1}$; $2.59 \pm 0.39\%$ for a concentration of $0.5 \mu\text{g.mL}^{-1}$; $3.065 \pm 0.24\%$ for a concentration of $1.25 \mu\text{g.mL}^{-1}$; $11.025 \pm 0.67\%$ for a concentration of $5 \mu\text{g.mL}^{-1}$; $19.19 \pm 1.08\%$ for a concentration of $12.5 \mu\text{g.mL}^{-1}$ and only $33.94 \pm 7.45\%$ for a concentration of methanolic leaf extract of $25 \mu\text{g.mL}^{-1}$. The anti-free radical activity of leaves is only $66.02 \pm 8.21\%$ for a concentration of

$50 \mu\text{g.mL}^{-1}$. The IC_{50} for methanolic extract of *W. indica* leaves is $37.5 \mu\text{g.mL}^{-1}$.

4. Discussion

In this study, we evaluated the anti-radical activity of methanolic and aqueous extracts of various organs of *W. indica* by scavenging the radical ion $\text{ABTS}^{\bullet+}$ according to the method of Re *et al.* [26] with gallic acid as the standard antioxidant. This very sensitive method optimized by N'negue *et al.* [27] is widely used for the determination of the antioxidant activity of plant extracts, hydrophilic and lipophilic compounds, red fruits etc. [28; 29].

The results of the evaluation of the anti-free radical activity of extracts of *W. indica* showed the presence of anti-free radical activity on all three organs tested, namely the stem, stem bark and leaves; which is in agreement with the literature [14; 20; 23; 24; 25]. Indeed, these authors have demonstrated the antioxidant activity of the whole plant, extracts of roots and leaves of *W. indica*.

However, our results show that anti-free radical activity varies with the organ and the method of extraction. In fact, the first comparison of the anti-free radical activity of the methanolic extracts of the different organs of *W. indica* shows that the stem bark is the most active organ, with an IC_{50} of $4.3 \mu\text{g.mL}^{-1}$, followed by the stem with an $\text{IC}_{50} = 6 \mu\text{g.mL}^{-1}$. The leaf is by far the least active organ with an IC_{50} of $37.8 \mu\text{g.mL}^{-1}$. In terms of percentage of anti-free radical activity, for the same concentration of methanolic extracts of $6.25 \mu\text{g.mL}^{-1}$, the anti-free radical activity is $70.08 \pm 5.96\%$ for the stem bark; $55.08 \pm 1.97\%$ for the stem and only $12.06 \pm 1.45\%$ for the leaf. For a concentration of $12.5 \mu\text{g.mL}^{-1}$, the anti-free radical activity is 86% for the stem bark and the stem and only 19.19% for the leaves. For concentrations of methanolic extracts of $25 \mu\text{g.mL}^{-1}$ and more (37.5 and $50 \mu\text{g.mL}^{-1}$) the anti-free radical activities of the stem bark and the stem reach 100% while for the leaf they remain below 100%. In fact, the anti-free radical activities of the methanolic extract of *W. indica* leaf are $33.94 \pm 5.45\%$; $49.75 \pm 2.63\%$ and $66.02 \pm 7.21\%$ for the concentrations of 25, 37.5 and $50 \mu\text{g.mL}^{-1}$ respectively.

From these results we can also deduce that for concentrations greater than its IC_{50} ($6 \mu\text{g.mL}^{-1}$) the stem has an anti-free radical activity equivalent to that of its bark. According to the literature, the whole plant of *W. indica* is composed of tannins and flavonoids which are polyphenolic compounds with antioxidant activity capable of scavenging free radicals; the leaves are composed of phenolic acids and tannins and the stem of tannins [22; 30]. It would appear from our results that the bioactive compounds responsible for the anti-free radical activity are either in greater quantity in the stem bark and the stem (which have little different IC_{50} s) than in the leaf; or that the bioactive compounds present mainly in the stem and its bark are more soluble in methanol than those present in the leaves. Indeed, the photochemical constituents of *W. indica* vary from organ to organ [12].

Gallic acid, a reference antioxidant, showed an IC_{50} of $0.41 \mu\text{g.mL}^{-1}$, i.e. an anti-free radical activity 10.5 times greater than that of methanolic stem bark extract, 15 times greater than that of the methanolic stem extract, and about 91 times greater than that of the methanolic extract of the leaves of *W. indica*. Given the pure character of gallic acid and the presence of non-active compounds in the methanolic extracts of *W. indica* reducing the concentration of the active principle present in these extracts, we can say that the methanolic extracts of the stem of *W. indica* and of its bark have antioxidant activities more or less equivalent to that of gallic

acid “reference antioxidant”.

The second step is to compare the anti-free radical activity and the mode of extraction on the results of the anti-free radical activities of the aqueous and methanolic extracts of *W. indica* stems (Figures 2 and 3). According to our results, the IC_{50} s are $2.5 \mu\text{g.mL}^{-1}$ for the aqueous extract of the stem and $4.3 \mu\text{g.mL}^{-1}$ for the methanolic extract. The aqueous extract of the stem is said to be about 2 times more anti-free radical than the methanolic extract. In terms of percentage of anti-free radical activity, the results obtained showed that for the same concentration of $0.05 \mu\text{g.mL}^{-1}$, the anti-free radical activity is $5.54 \pm 0.55\%$ for the aqueous extract and $1.58 \pm 0.28\%$ for the methanolic extract, a difference of about 4%. For a concentration of $0.5 \mu\text{g.mL}^{-1}$, the anti-free radical activity is $8.495 \pm 0.09\%$ for the aqueous extract and $4.72 \pm 0.88\%$, a difference of 3%. For a concentration of $0.625 \mu\text{g.mL}^{-1}$, the anti-free radical activity is $12.4 \pm 0.52\%$ for the aqueous extract and $8.64 \pm 1.88\%$, a difference of approximately 4%. With a concentration of $5 \mu\text{g.mL}^{-1}$ we obtained an anti-free radical activity of $57.14 \pm 0.54\%$ for the aqueous extract and of $42.66 \pm 3.99\%$ for the methanolic extract, a difference about 10% taking into account the standard deviation. For a concentration of $12.5 \mu\text{g.mL}^{-1}$ the anti-free radical activity is $91.82 \pm 1.22\%$ for the aqueous extract and $86.41 \pm 1.08\%$ for the methanolic extract, a difference 5.5%. Finally for concentrations of 25; 37.5 and $50 \mu\text{g.mL}^{-1}$ the anti-free radical activity is 100% on average for the aqueous and methanolic extracts. We can therefore say from these results that the aqueous stem extract has an anti-free radical power slightly greater than the methanolic stem extract, between 3 and 10%.

The bioactive compounds responsible for the anti-free radical activity of the stem, namely the tannins^[12] seem to be a little more soluble in water than in organic solvents, which would explain the somewhat greater quantity of these bioactive elements. in aqueous solvent than in methanol for concentrations below $12.5 \mu\text{g.mL}^{-1}$. Other authors have found greater antioxidant activity in an aqueous extract of a plant compared to its methanolic extract^[31]. This is the case with the aqueous extract of the roots of *Atractylis gummifera* (Glue Coal) which exhibits greater antioxidant activity than that of its methanolic extract^[31]. On the other hand, if we compare the anti-free radical activity of the aqueous extract of the stems of *W. indica* “ $IC_{50} = 2.5 \mu\text{g.mL}^{-1}$ ” with that of gallic acid, a reference antioxidant “ IC_{50} of $0.35 \mu\text{g.mL}^{-1}$ ” we can say gallic acid has an anti-free radical activity only 7 times greater than that of the aqueous stem extract against 17 times greater for the methanolic stem extract. In the search for natural antioxidants an aqueous extraction of these bioactive compounds would be more efficient than a methanolic extraction for the stems of *W. indica*. In fact, the anti-free radical activity of the aqueous extract of the stems is far superior to that of the methanolic extract of the bark of the stem, the most antioxidant organ. It would be interesting to see what happens for the aqueous extracts of the other organs of *W. indica* evaluated here namely the leaves and stem bark; and not rated such as flower, seed or fruit. Indeed, the leaves of *W. indica* being also composed of phenolic acids^[12], hydrophilic compounds with antioxidant character, their high concentration in an aqueous extract could greatly increase the anti-radical activity of an aqueous extract of the leaves.

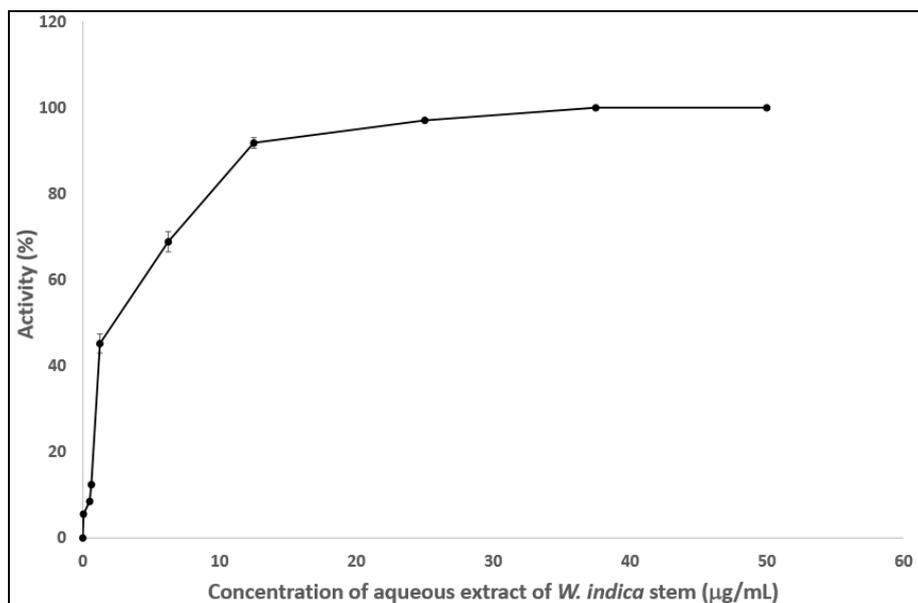


Fig 1: Anti-free radical activity of the stems aqueous extract of *W. indica* after 6 minutes of incubation. The proportion $ABTS^{\bullet+}$ transformed into $ABTS^+$ in the presence of the stems aqueous extract is calculated from the change in absorbance at 734 nm measured by spectrophotometry; $n = 3$.

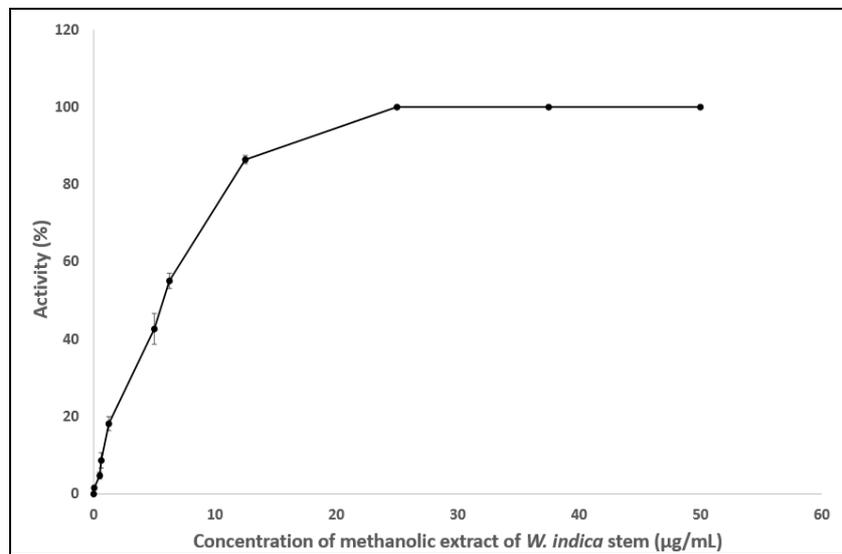


Fig 2: Anti-free radical activity as a function of the concentration of methanolic extract of *Waltheria indica* stems after 6 minutes of incubation. The proportion $ABTS^{\bullet+}$ transformed into $ABTS^+$ is calculated from the change in absorbance at 734 nm measured by spectrophotometry; $n = 3$.

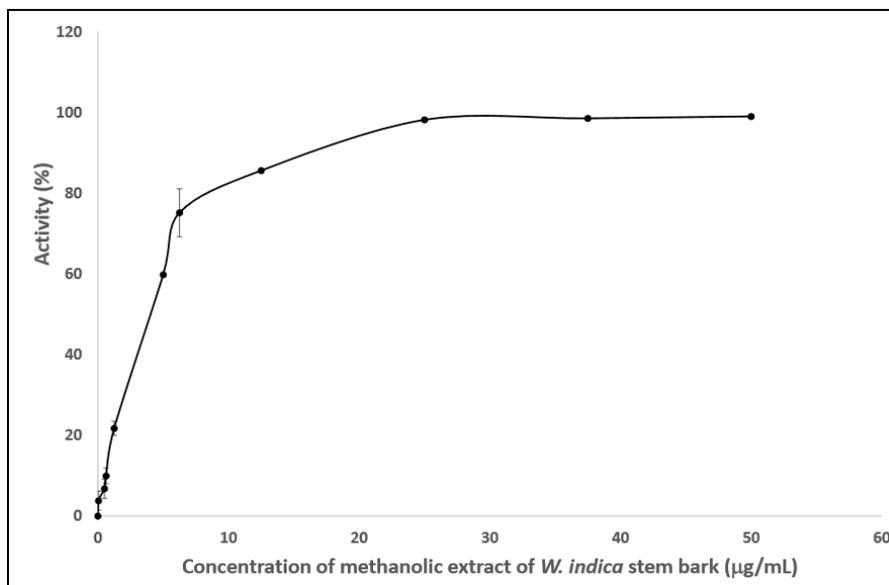


Fig 3: Anti-free radical activity as a function of the concentration of methanolic extract of *W. indica* stem bark after 6 minutes of incubation. The proportion $ABTS^{\bullet+}$ transformed into $ABTS^+$ is calculated from the change in absorbance at 734 nm measured by spectrophotometry; $n = 3$.

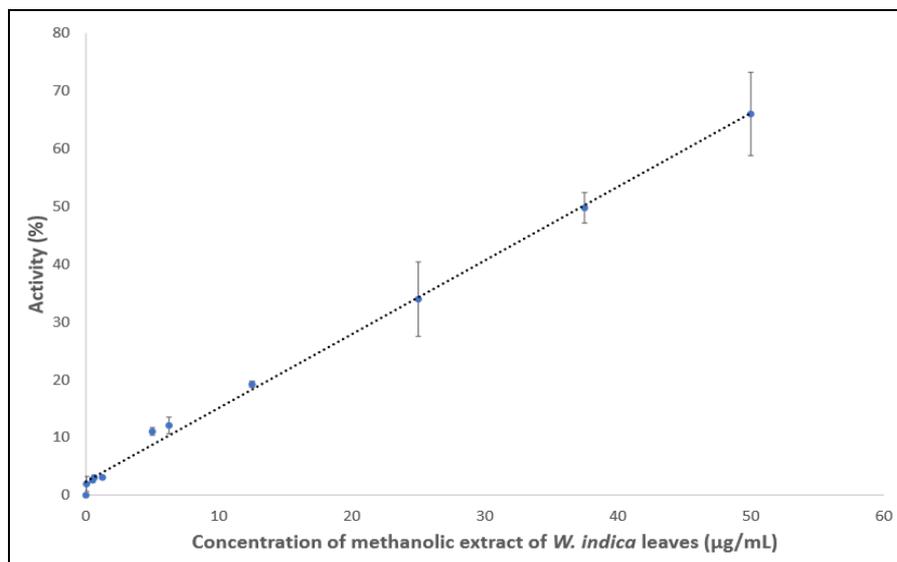


Fig 4: Anti-free radical activity as a function of the concentration of methanolic extract of *Waltheria indica* leaves after 6 minutes of incubation. The proportion $ABTS^{\bullet+}$ transformed into $ABTS^+$ is calculated from the change in absorbance at 734 nm measured by spectrophotometry. The equation on the curve is: $y = 1.277x + 2.32$ ($R^2 = 0.97$); $n = 3$.

5. Conclusion

The various organs of *W. indica*, namely the stem, stem bark and leaves, from Gabonese soil are endowed with anti-free radical activity. The stem bark is the most active organ followed by the stem. The leaf is the part of the plant that showed the least activity. However, an effect of the mode of extraction of bioactive compounds on the anti-free radical activity of the organ has been observed. Thus, the anti-free radical activity of the aqueous stem extract was superior to that of the methanolic stem bark extract. Given the harmful effects of oxidation reactions in the body, food and cosmetics; the various organs of *Waltheria indica*, particularly the stem bark, can provide natural antioxidants that can be used in preventive medicine and in the food and pharmaceutical industry.

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