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# Phytochemical analysis and *in vitro* antioxidant assay of crude ethanol extract of *Justicia secunda vahl* leaves

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**Abstract**
*Justicia secunda* is a plant with diverse range of applications in African traditional medicine. Its therapeutic tendency in anemia is rooted in native traditions. Further scientific research is warranted to validate the efficacy of *J. secunda* as a valuable resource for alternative and complementary medicine. The present study evaluated the total phenolic, total flavonoid content and *In-vitro* antioxidant activity of *J. secunda* leaves ethanol extract. The extraction was performed by cold maceration method. 100 g (0.1 kg) of the powdered leaf of *J. secunda* was weighed and macerated in 500 mL of 95% ethanol for 48 hours. The mixture was agitated each day at regular intervals to ensure homogenous extraction at room temperature. Total phenolic and flavonoid content assay were performed using standard protocols. The extract was screened for antioxidant activity with ascorbic acid as reference standard. The extract showed the presence of phenolic and flavonoid contents. The extract also showed *In-vitro* antioxidant activity with DPPH model having $IC_{50}$ values of 1.105 µg/mL (ethanol extract) and 1.634 µg/mL (ascorbic acid) and the TAC model having $IC_{50}$ values of 1.103 µg/mL (ethanol extract) and 1.634 µg/mL (ascorbic acid). The research demonstrates that the ethanol extract of *J. secunda* leaves possess significant antioxidant activity due to its ability to scavenge free radicals and inhibit oxidative processes. In conclusion, the ethanol extract of *J. secunda* leaves represents a rich source of phenolic and flavonoid compounds with notable antioxidant potential. These findings support its therapeutic potential and encourage further research into its specific bioactive components and broader pharmaceutical applications.

**Keywords:** $IC_{50}$, *Justicia secunda*, blood, anti-oxidant, anemia

## 1. Introduction
A medicinal plant is any plant that, in one or more of its organs, contains substances that can be used for therapeutic purposes or precursors for synthesizing useful drugs [1, 2]. The many years of struggles against illnesses prompted mankind to explore the use of medicinal plants and plant parts such as barks, seeds and fruit bodies as ameliorative strategies [3]. Despite the overall improvement in general healthcare delivery, the role of medicinal plants in animal and human health care systems cannot be over emphasized, as about 60% of world population still depend on herbs for their primary health care [4, 5]. *Justicia secunda Vahl* is a creeping evergreen perennial herbal plant, which belongs to the family *Acanthaceae*, order *Scrophulariales* and superorder *Lamiflorae* [6, 7]. Its species are widespread in tropical regions of the world and are poorly represented in temperate regions. Its species are found in Asia, America, Africa, and South America. In Africa, they are cultivated majorly in Nigeria, Ghana, Gabon and the Democratic Republic of the Congo (DRC). It's also known as *"Lamiaceae*" and "*Justicia adhatoda*" [6]. *J. secunda Vahl* is commonly known as "Bloodroot" (which refers to the red color of water observed when the plant is boiled) [7, 8]. It is known as "Obara Bundu or Ogwu Obara" by the Igbo. The Yoruba call it "Ewe eje" (Blood leaf) or "Ewe ajeri" (Jehovah's Witness leaf) and "Hounsiman" in Benin [7]. The folkloric uses of the plant include wound healing, anemia and abdominal pain [9, 10] In Nigeria, Congo and South Cote-d'Ivoire the leaf brew is consumed by Jehovah's Witness believers in the management of anemia [11]. Phytochemicals are secondary metabolites of low-molecular weight that are produced by plants in discrete quantity. Phytochemicals that have or seem to possess significant effect on human health are grouped as carotenoids, phenolic compounds (flavonoids, phytoestrogens, and phenolic acids), phytosterols and phytostanols, tocotrienols, organosulfur compounds (allium compounds and glucosinolates) [12].

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant Defenses [13]. Oxidative stress is well known to be involved in the pathogenesis of lifestyle-related diseases, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and malignancies [14]. Antioxidants are substances that can scavenge free radicals and help to decrease the incidence of oxidative stress-induced damage. Traditional herbal medicines, and dietary foods were the main sources of antioxidants for ancient peoples that protected them from the damage caused by free radicals. Phytochemicals have been shown to exert their positive antioxidant benefits in animal models ranging from increased general performance to production quality and enhanced endogenous antioxidant system, possibly by directly modifying specific molecular mechanisms or indirectly as stabilized conjugates altering the metabolic pathway [15]. As such, this study aims to dissect the phytochemical composition and *in vitro* antioxidant assay of crude ethanol extract of the leaves of *Justicia secunda.*

## 2. Materials and Methods

### 2.1 Procurement and identification of the plant

The leaf of the plant was collected from Elele, River's state, Nigeria in the month of June 2023. A voucher specimen (voucher number MU/PHGSY/) of the plant was deposited at the herbarium for Phytotherapy, Faculty of Basic Medical Sciences, Madonna University, Nigeria.


**Fig 1:** *Justicia secunda* plant.

### 2.2 Chemicals and reagents

The chemical ethanol was produced from Guandong Guanhua Sci-tech, Shantou, China. DPPH was obtained from Sigma-Aldrich Steinheim, Germany. All chemicals and solvents used were of analytical grades

### 2.3 Extract preparation

Fresh matured leaves of *J. secunda* were collected and dried in open air for days. The dried leaves were further pulverized into powdered particles for the purpose of this study. The extraction was performed by cold maceration method.100g (0.1kg) of the powdered leaf of *Justicia secunda L*. was weighed out and macerated in 500ml of 95%ethanol for 48hours.The mixture was agitated each day at regular intervals to ensure good and complete extraction. Thereafter, the mixture was pre-filtered using Mushin cloth after which the filtrate was refiltered using a whats-mann filter paper. The filtrate was concentrated by oven evaporation at room temperature.

### 2.4 Quantitative phytochemical analysis of the ethanol extract of *Justicia secunda L.*

Stock solution of sample was prepared by dissolving 100mg of extract in 100mL of distilled water (1mg/mL).

#### 2.4.1 Determination of Total Phenolics Content (TPC)

Folin-Ciocalteau method was used for the determination of the total phenolics content of the extracts using gallic acid as an internal standard with slight modification. Briefly, the extract (1 mg/mL) was mixed with distilled water (9 mL) in a 20 mL volumetric flask. Two and half milliter (0.4 mL) of a 10-fold dilute Folin-Ciocalteau phenol reagent (FCPR, 1:10) was added. After 5 minutes 4 ml of 7.5% of sodium trioxocarbonate (IV) ($Na_2CO_3$) solution was added to the mixture and made up to the mark with distilled water. The mixture was incubated in the dark for 90 minutes at room temperature. A set of standard solutions of gallic acid (20, 40, 60, 80, 100 µg/mL) were prepared in the same manner as described for the extracts. The absorbance of the extract and standard solutions were read against the reagent blank at 765 nm with a UV/Visible spectrophotometer (UV-1800, Shimadzu, Japan). The determination of the total phenolics in the extracts was carried out in triplicate. The Total Phenolics content was determined from the calibration curve and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract [16]

#### 2.4.2 Determination of Total Flavonoid Content (TFC)

Aluminium-Chloride colourimetric assay was used to determine the total flavonoids content in the extract as previously reported [17]. Briefly, 1 mL of the extract (1 mg/mL) was mixed with 4 mL of distilled water in 20 mL volumetric flask. 0.30 mL of 5% sodium nitrite was added to the flask. After 5 minutes, 0.30 mL of 10% AlCl3.6H2O solution was added to the mixture, followed by addition of 2 mL of 1.0 M sodium hydroxide (NaOH) after another 5 minutes and diluted to the mark with distilled water. A set of standard solutions of quercetin (20, 40, 60, 80, 100 µg/mL) were prepared in the same manner as described for the extracts. The absorbance of the extract and standard solutions were read against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The determination of total flavonoids in the extract and standards were carried out in triplicates. The total flavonoids content was expressed as milligram of quercetin equivalent (QE) per gram of extract.

### 2.5 *In vitro* antioxidant assays of the ethanol extract of *Justicia secunda L*

The *in vitro* antioxidant assays of the extract were carried out by dissolving 0.2 g of the extract in 10 mL of distilled water and then 1 in 20 dilutions to form stock solutions of 1 mg/mL (1000 µg/mL). Serial dilutions (15.63, 31.25, 62.5, 125, 250, 500, 1000 µg/mL) of each extract were made from the stock solution. Ascorbic acid (AsA) and Gallic acid were used as standard for the antioxidant assays.

#### 2.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The radical scavenging activity of the extract and fractions was determined by the DPPH assay with slight modifications [18, 19]. A fresh stock solution of DPPH was first prepared by dissolving 4.5 mg of DPPH in 100 mL of ethanol. A volume, 1 mL of sample solution and 3 mL of DPPH stock solution were mixed, and was incubated at room temperature for 30 minutes in the dark before absorbance was read at 517 nm.

The DPPH radical scavenging activity of ascorbic acid was also determined for comparison and all tests were performed in triplicate. The inhibition percentage (%) of radical scavenging activity was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times \frac{100}{1}$$

**2.5.2 Total Antioxidant Capacity (TAC) assay using phosphomolybdate method**

The total antioxidant capacity assay of the extract was carried out by the Phosphomolybdate method as previously reported [20]. Briefly, 0.1 mL aliquot of different concentrations (15.63, 31.25, 62.5, 125, 250, 500, 1000 µg/mL) of the extract, fraction and ascorbic acid was mixed with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate, 1:1:1). The test tubes were covered with aluminum foil and incubated in a water bath at 95 °C for 90 minutes. After the extract was cooled to room temperature, the absorbance of the mixture was determined at 765 nm against a blank containing 1 mL of the reagent solution. Ascorbic acid was used as standard. The assay was carried out in triplicate. The antioxidant capacity (TAC) is expressed as milligram Ascorbic acid equivalent per gram of the extract (mgAAE/g). The antioxidant capacity was estimated using the following formula:

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Absorbance of control - Absorbance of extract}}{\text{Absorbance of control}} \times \frac{100}{1}$$

**Statistical analysis**

The values of all the parameters determined are expressed as Mean ± SEM where n=3. The level of significance was placed at $p<0.05$. All statistical analyses were performed using SPSS version 21.0.

## 3. Results and Discussion

### 3.1 Percentage yields of Extract

**Ethanol extract**

Weight of fresh leaves 100 g
Volume of ethanol used for extraction 500 mL of 95% ethanol
Weight of empty bottle 7.54 g
Weight of dried ethanol extract in bottle 10.40 g
Mass of ethanol extract is given as 10.40g-7.54g = 2.86 g
Percentage yield is given as% yield = 2.86/100*100 = 2.86%

Extraction is crucial to isolate active compounds from plant materials and reduce interferents [21]. It has been reported that ethanol extracts of Ivorian plants extracted higher concentrations/amount of phenolics compared to acetone, water and methanol [22]. The result showed that when extracted with 500 mL of 95% ethanol 100g of the dried *J. secunda* leaves produced 2.86g of extract indicating a percentage yield of 2.86%

### 3.2 Result of Quantitative Phytochemical Analysis of Ethanol extract of *J. secunda*

**Table 1:** Result of total phenolic content of *J. secunda*

| Conc (µg/ml) | TPC of Extract (mg/g GAE) |
|---|---|
| 25 | 12.32± 0.04 |
| 50 | 44.12±0.05 |
| 100 | 98.10±0.21 |
| 200 | 132.22±1.00 |
| 250 | 300.12±1.04 |
| 300 | 415.18±1.06 |

Results expressed in Means ± SEM (N=3).

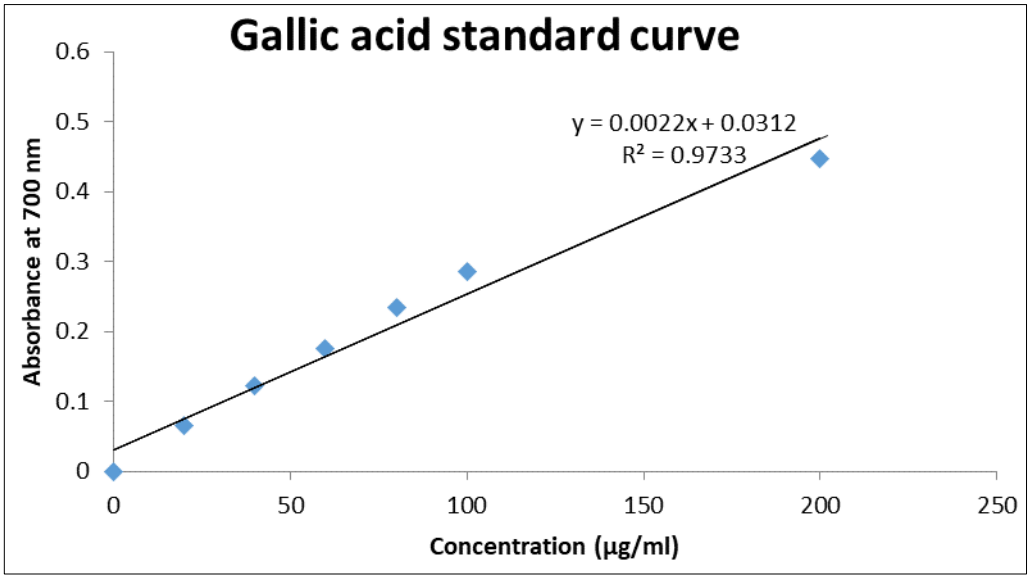
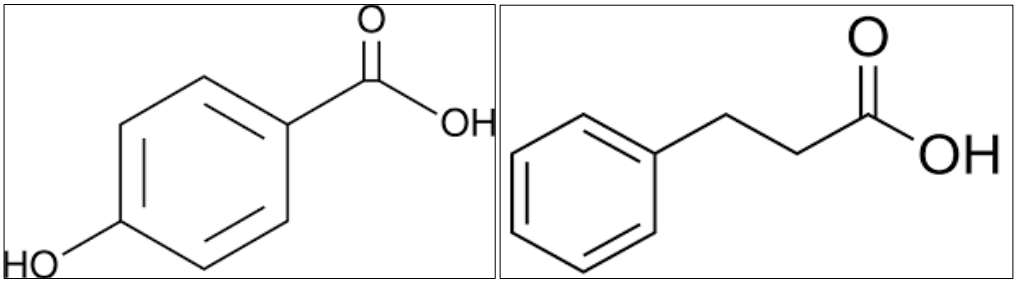



**Fig 1:** Absorbance concentration graph for total phenolic content

Total phenolic contents of different concentrations of the extracts were determined by Folin-Ciocalteau method using gallic acid as the standard. The absorbance values obtained at different concentrations of gallic acid were used for the construction of calibration curve. Total phenolic content of the extracts was calculated from the regression equation of

calibration curve ($Y$ = 0.0022x + 0.0312; $R^2$ = 0.9733) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g)

TPC is one of the important parameters of total antioxidant capacity (TAC) and is widely used for the evaluation of the antioxidant properties of plants [23]. Due to the plant's diverse phenolics and antioxidant substrates with diferent structures, molecular sizes, and polarities, the nature and concentration of extraction solvents can greatly affect their results [24]. TPC values increased proportionally with an increase in concentration of the extract up to 300µg/ml. Indicating positive correlation between the concentration of ethanol extract of *J. secunda* and the total phenolic content. Studies by (Osioma & Hamilton-Amachree, 2017) [25] and (Jimoh, *et al*., 2023) [26] have shown that ethanol extract of *J. secunda* plant contains phenolic compounds




4-Hydroxybenzoic acid Hydroxycinnamic acid

**Fig 2:** Chemical structures of some phenolic acid compounds.

### 3.2.1 Result of Total Flavonoid Content of *J. Secunda*

**Table 2:** Results of Total Flavonoids Content (mgQE/g) of *Justicia secunda L.*

| Conc (µg/ml) | TFC of extract (mgQE/g) |
|---|---|
| 25 | 16.15±0.34 |
| 50 | 52.23±1.00 |
| 100 | 114.53±1.02 |
| 200 | 148.20±1.25 |
| 250 | 311.20±0.71 |
| 300 | 472.10±0.03 |

Results expressed in Means ± SEM (N=3).

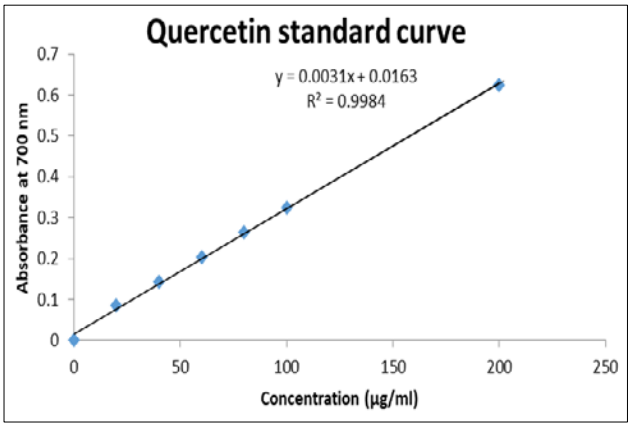



**Fig 3:** Absorbance concentration graph for total flavonoid content.

The total flavonoid in the crude extracts and standard was measured following the $AlCl_3$ colourimetric assay using quercetin as the standard. The absorbance values obtained at different concentrations of quercetin were used for the construction of calibration curve. Total flavonoid content of the extracts was calculated from the regression equation of calibration curve ($Y$ = 0.0031x + 0.0163; $R^2$ = 0.9984) and expressed as milligram of quercetin equivalent (QE) per gram of extract.

Similarly to TPC, TFC values also increased proportionally with an increase in concentration. As 300µg/ml of the ethanol extract produced 472.10±0.03 mgQE/g of flavonoids. Indicating positive correlation between the concentration of ethanol extract of *J. secunda* and the total flavonoid content.

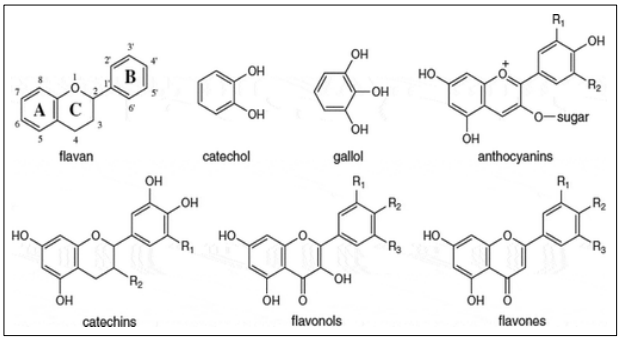



**Fig 4:** Chemical structures of some common flavonoids

**Table 3:** Result of antioxidant activity of *j.secunda* by DPPH scavenging free radical capacity

| Varying concentration (ug/mL) | Extract | Ascorbic acid |
|---|---|---|
| 15.63 | 66.16±0.05 | 56.02±1.02 |
| 31.25 | 72.00±0.12 | 57.14±0.12 |
| 62.5 | 78.16±0.07 | 60.02±0.27 |
| 125 | 84.22±0.05 | 62.18±0.01 |
| 250 | 88.12±0.22 | 64.00±0.12 |
| 500 | 91.02±0.11 | 66.16±0.34 |
| 1000 | 92.56±0.11 | 67.22±0.20 |
| $IC_{50}$ | 1.105 | 1.634 |

### 3.2.2 Results expressed in Means ± SEM (N=3)

DPPH is one of the free radicals widely used for testing the preliminary antioxidant activity of plant extracts. The decrease in absorbance of DPPH solution is proportional to the antioxidant concentration [27]**.** The result of the DPPH photometric assay of *J. secunda* is presented in Fig 3.3. *J. secunda* extract caused a concentration dependent increase in percentage antioxidant activity increasing the antioxidant activity from 66.16% at 15.63 µg/ml concentrations to 92.56% at 1000 µg/ml concentrations while ascorbic acid had 67.22% at 1000 µg/ml concentrations.

The observed high antioxidant scavenging properties in *J. secunda* leaf extract could be attributed to the higher concentrations of flavonoids and phenols, in the leaf extract of the plant. The antioxidant activities as exhibited by the plant's ability to scavenge DPPH coupled with the phytochemicals contained in the leaf of *J. secunda* could justify its use locally for medicinal purposes [25]

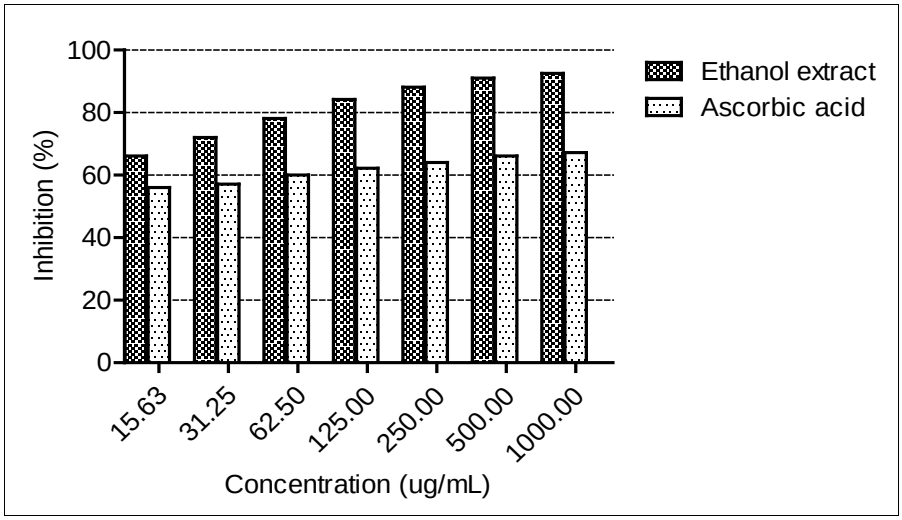



**Fig 5:** DPPH scavenging activity of ethanol extract against ascorbic acid

**Table 4:** Result of TAC (The total antioxidant capacity) of *J. secunda*

| Varying concentration (ug/mL) | Extract | Ascorbic acid |
|---|---|---|
| 15.63 | 66.16±0.05 | 56.02±1.02 |
| 31.25 | 72.00±0.12 | 57.14±0.12 |
| 62.5 | 78.16±0.07 | 60.02±0.27 |
| 125 | 84.22±0.05 | 62.18±0.01 |
| 250 | 88.12±0.22 | 64.00±0.12 |
| 500 | 91.02±0.11 | 66.16±0.34 |
| 1000 | 92.56±0.11 | 67.22±0.20 |
| IC50 | 1.105 | 1.634 |

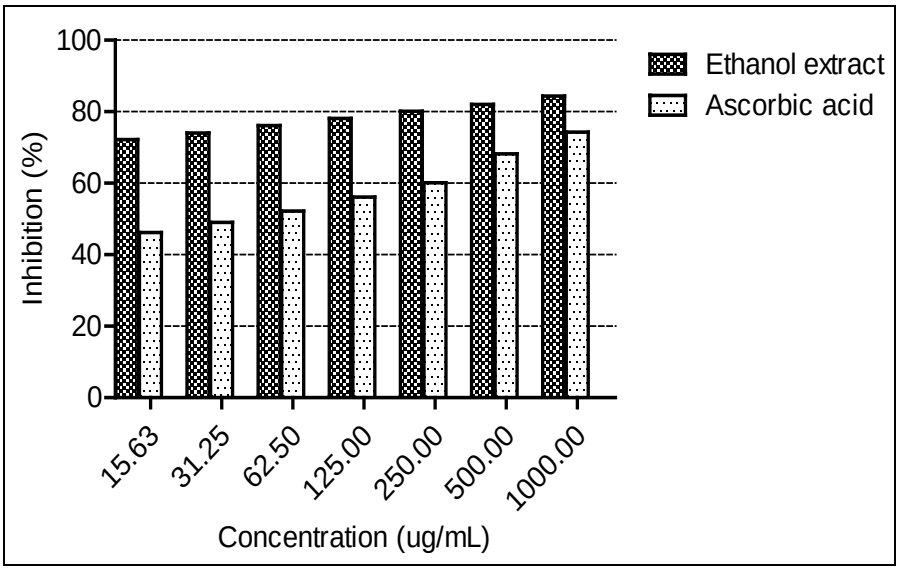



**Fig 6:** Total antioxidant activity of ethanol extract against ascorbic acid.

The figure above indicates the total antioxidant capacity of the ethanol extract against ascorbic acid. At the concentration of 15.63 µg/ml the total antioxidant capacity of *J. secunda* extract was 72.24% and at 1000 µg/ml it was 84.35% while ascorbic acid had 46.22% and 74.25% at 15.63 µg/ml and 1000 µg/ml concentrations respectively.

The result of this study shows that the ethanol extract possesses more considerable free radical scavenging activities compared to ascorbic acid possibly due to the presence of various phytochemicals such as flavonoids, tannins and phenols [28, 29]. It was also observed that the free radical scavenging activity exhibited by extract increased concurrently with increase in concentration. The half maximal inhibitory concentration ($IC_{50}$) was obtained. The $IC_{50}$ is a measure of the potency of a substance in inhibiting a specific biological function. Lower $IC_{50}$ indicates a higher antioxidant activity as seen in ethanol extract compared to the ascorbic acid (standard) using both models [30]

## 4. Conclusion

The Ethanol extract of *Justicia secunda* was shown to possess numerous phytochemicals that are responsible for the medicinal plant's antioxidant activities. The increase in TPC and TFC values with increase in concentration of the extract indicates the potency of the plant in terms of combating oxidative stress. The DPPH and TAC assay further reveal the antioxidant potential of the ethanol extract of *J. secunda*. It is recommended that more studies should be done to ascertain

other therapeutic effects of *J. secunda* and also determine the potency of other extraction solvents as this will reveal more efficient extraction techniques to obtain more phytochemicals from the plant.

**Conflict of interest**

The authors declared no conflict of interest.

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