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Phytochemistry and Antioxidants Activities of Four Different Solvent Extracts of *Justicia secunda* Stem.

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Abstract
This study was aimed to evaluate phytochemical and antioxidant activities of *Justicia secunda* stem crude extracts from four different solvents in increasing polarities, with the idea of isolating some of the metabolites (secondary). In order to ensure that a wide range of polarities of the compound could be extracted, the method of cold maceration was used in the extraction by serial exhaustive extraction method which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol). The crude extracts were concentrated using rotary evaporator at 40^0C; it was observed that methanol extract had the highest yield (4.13 %); followed by acetone (2.40 %); ethyl acetate (1.65 %) and hexane had the least yield (1.03 %). Phytochemical screening of the crude extracts showed the presence of alkaloids, flavonoids, terpenoids, tannins, phenol, steroids cardiac glycoside and phlobatannins The antioxidant activity of the crude extracts were determined using DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay at various concentrations of 0.0313, 0.0625, 0.1250, 0.2500, and 0.500mg/mL, with vitamin C as standard. The IC_{50} values were calculated and observed that the standard had the least IC_{50} value of 22.762 mg/mL indicating highest scavenging ability, comparing to that of the extracts, methanol extract (48.04 mg/mL) was closely comparable to that of the standard, closely followed by that of acetone extract (53.71 mg/mL), then ethyl acetate (66.03 mg/mL), and lastly hexane (74.62 mg/mL).

Keywords: Phytochemicals, antioxidant activity, medicinal plants, *Justicia secunda,* DPPH, free radicals scavenging activity.

Introduction

For thousands of years, nature has been a source of medicinal agents. Typically, nature produces a lot of secondary metabolites, which are crucial for the development of new environmentally friendly microbicides, pesticides, herbicides, and many pharmaceutical drugs [1]. There are two broad categories that can be applied to all biological system compounds. One is primary metabolites, which are chemicals like carbohydrates, amino acids, proteins, and lipids that promote growth and development [2]. Another is the group of compounds known as secondary metabolites, which, in addition to primary metabolites, are thought to enable plants to interact with their surroundings, thereby enhancing their overall capacity for survival and overcoming local obstacles [3]. Men turned to ethno-pharmacognosy as a result of the development of adverse effects and microbial resistance to chemically synthesized medications. They discovered literally thousands of phytochemicals from plants that were safe, generally effective, and had fewer side effects [4]. There were numerous reports of beneficial biological activities, including anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic, and wound healing activity. Numerous disorders and diseases are caused by reactive oxygen species (ROS) and other oxidants, as shown by substantial evidence. Scientists now recognize the value of antioxidants for disease prevention, treatment, and human health maintenance thanks to the evidence [5].

The plant *Justicia secunda* is a restorative plant which has a place with the acanthaceae variety, its species are far and wide in tropical locales of the world and are ineffectively addressed in calm districts. Asia, the United States, and Africa all contain it. In Barbados, it is commonly referred to as blood plant [6]. Studies attempting to provide scientific support for the traditional uses of this plant have revealed a variety of its ethno-medicinal significance. The activity that lowers blood pressure [7], and the anti-hyperglycemic and hypoglycemic effects [8]. *Justicia secunda* is one of the plants which have been used in traditional medicine for many years. To the best of our knowledge little or no work has been done on the plant *Justicia secunda* in Taraba, Nigeria. This work is designed to enrich the available scientific data on the phytochemistry and antioxidant activity of *Justicia secunda* stem.

Materials and Methods

Sample collection and preparation

The stem of *Justicia secunda* were collected from their natural habitat in Wukari Local Government Area of Taraba State, Nigeria. The fleshly collected stems of *Justicia secunda* was washed with distilled water, cut into smaller pieces then air-dried in a ventilated room for three weeks. After drying, the stem were pounded into powder form with a mortar and a pestle. The powdered plant materials were reserved in sealed containers for extraction.

Method of extraction

The method of cold maceration was used in the extraction by serial exhaustive extraction method which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. The extracts of the stem were prepared by soaking 100 g of each in 250 ml hexane for four days with frequent agitation until soluble matter is dissolved. The resulting mixture was filtered by gravity filtration and the filtrate to be concentrated by evaporation using rotatory evaporator and weighed. The procedure was repeated on the residue using the following solvents: ethyl acetate, acetone and methanol sequentially in order of polarity. The extracts were stored in a refrigerator under argon condition until required for testing. The yield and percentage yield of the extracts was calculated. The yield and percentage yield of extracts were calculated as a ratio of the original sample as shown below:

$$\text{Percentage of yield } \% = \frac{\text{Weight of Extraction}}{\text{Weight of dried fraction}} \times 100 \quad (1)$$

Thus,

$$\text{Yield of Extract} = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \quad (2)$$

Where: W_1= weight of conical flask, W_2 = weight of conical flask + sample, and W_3 = weight of conical flask + Extract

Phytochemical screening assay

This is a qualitative analysis, which involves performing some simple chemical test in order to detect the kinds of natural products that may be present in the plant extract (i.e. secondary metabolites). Phytochemical examinations were carried out for all the extracts using standard procedures to identify the constituents. Some of the natural products include alkaloids, saponins, reducing sugars, phlobatannins, tannins, flavonoids, glycosides, cardiac glycosides, terpenes, steroids and phenol. Qualitative analysis of the crude extracts was carry out as described by [9] and [10] to identify the presence of the classes of secondary metabolites (alkaloids, anthraquinones, flavonoids, tannins, saponins, glycosides, cardiac glycosides, terpenes, steroids, phenol, etc).

Test for alkaloids

Crude extract (0.5 g) was stir with 2 M aqueous hydrochloric acid (5.0 mL) on a steam bath and filter.

a) Mayer's test

Filtrates (1.0 mL) of the extracts was treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

b) Wagner's test

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

c) Dragendroff's test

Filtrates will be treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

d) Hager's test

Filtrates will be treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids will be confirmed by the formation of yellow coloured precipitate.

Test for tannins

Plant extracts (0.5 g) will was boiled with distilled water (100 mL) for 5.0 minutes. To 2.0 cm^3 of the cooled solution (filtrate) a few drops of ferric chloride was added and observed for brownish green or a blue black colouration.

Test for phlobatannins

An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid. Disposition of red precipitate determines the presence of phlobatannins

Test for Cardiac Glycosides

(a) Keller Killani test:

The extract (0.5 g) was dissolved in glacial acetic acid (2.0 mL) containing one drop of ferric chloride solution. A test tube was tilted and 1.0 cm^3 of conc. H_2SO_4 was added. A brown ring at the interface indicated the presence of a characteristic of cardenolides.

(b) Salkowski test:
The extract (0.5 g) was dissolved in chloroform (2.0 mL). Three drops of conc. H_2SO_4 was carefully added to form a lower layer. The change in the reaction was observed and then recorded.

Test for saponins
A small portion of each of the plant extracts was added to distilled water (20 mL) in a 100 mL beaker, boil and filter and the filtrate was used for the test.
(a) Froth test: A 5 20 mL of the filtrate was diluted with water (20 mL) and shake vigorously and allowed to stand for 30 minutes. Formation of 1 cm layer of foam will indicates the presence of saponins.

(b) Foam test: A 0.5 gm of extract was shaken with 2 mL of water. If foam produced persists for ten minutes it indicates the presence of saponins.

(c) Emulsion test: An olive (2 drops) was added to the frothing solution and shake vigorously. The result was recorded. In order to remove 'false-positive" the blood haemolysis test is to perform on the extract that frothed water.

Test for anthraquinones
The extract (0.5 g) was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10 % NH_3 solution was added to the mixture and heat. Formation of rose-pink colour indicates the presence of anthraquinones.

Test for flavonoids (Alkaline reagent test)
Extracts was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which became colourless on addition of dilute acid, indicates the presence of flavonoids.

Test for terpenoids (Salkowski test)
A solution of each of the extract was made by dissolving 0.5 g of the extract in 2.0 mL of chloroform and concentrated H_2SO_4. The presence of terpenes in the sample was detected by the presence of reddish-brown colouration of the interface as the colour changes.

Test for steroids
To the solution of the plant extract, acetic anhydride (3.0 mL) was added, mixed gently and allowed to cool in ice. This was followed by careful addition of a few drops of concentrated H_2SO_4. There was a colour changed from violet to blue indicating the presence of steroids.

Test for phenols
Extracts was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Quantitative determination of the chemical constituent
Quantitative determination of the detected secondary metabolites was carried out to know their percentages in the *Justicia secunda* stem by the methods described by [11], [12] and [13].

Detection of alkaloids
The stem sample (0.5g) was dissolved in 96 % ethanol- 20 % tetraoxosulphate(vi)acid(1:1) 1ml of the filtrate was added to 5ml of 60 % tetraoxosulphate(vi) acid and allowed to stand for 5mins. Then 2 mL of 0.5 % formaldehyde was added and allowed to stand for 3 hrs. The reading was taken at absorbance of 565 nm.
Detection of flavonoids
The determination of flavonoids on the stem sample was done by acid hydrolysis of spectrophotometric method. 0.5g of processed stem sample was mixed with 5ml of dilute hydrochloric acid and boiled for 10mins. The boiled extract was allowed to cool and filter 1ml of the filtrate was added to 5ml of ethyl acetate and 5 mL of 1% ammonium hydroxide. This was the scan from 420 – 520 nm for the absorbance.

Detection of saponin
The sample (0.5g) was added to 20 ml of 1NHCL and was boiled for 4hrs. After cooling it was filtered and 50 mL of pet ether was added to the filtrate for ether layer and evaporated to dryness. 5 mL of acetone-ethanol was added to the residue. 0.4 mL of each was take into three different test tubes, 6 mL of ferric sulphate reagent was added into them followed by 2 mL conc. Tetraoxosulphate(vi)acid. It was thoroughly mixed after 10 mins and the absorbance was taken at 490 nm.

Detection of phenols
The quantity of phenol is determined using the spectrophotometer method. The stem sample is boiled with 50 mL of pet spirit for 15 mins. 5mL of the boiled sample is then pipette into 50 mL flask and 5 mL of distilled water is added. After the addition of distilled water 2 mL of ammonium hydroxide solution and 5 mL of butanol is added to the mixture. The stem sample was made up to mark and left for 30 mins to react for colour development and measure at 505nm wavelength using a spectrophotometer.

Detection of tannins
The quantitative of tannins is determined by using spectrophotometric method. 0.5g of the stem sample is weigh into plastic bottle. 50 mL of distilled water is added and stirred for 1 hr. The sample is filtered into 50 mL volumetric and made up to mark. 5 mL of the filtered sample was then pipette out into test tube and mixed with 2 mL of

0.1 M HCl and 0.008M $K_4Fe(CN)_6.3H_2O$. The absorbance is measured with spectrophotometer at 395 nm wavelength within 10minutes.

Antioxidant assay using DPPH (1, 1-diphenyl-1-picrylhydrazyl)

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH [14]. About 0.1mM of DPPH in ethanol was prepared and 1ml of this solution was added to 3.0 mL of extract solution in ethanol at different concentrations (0.5, 0.25, 0.125, 0.0625, 0.03125 mg/mL). Thirty minutes (30) later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The same experiment was carried out on ascorbic acid which is known antioxidant. All test and analysis were run in duplicate and the results obtained were averaged. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\text{Inhibition } (\%) = \frac{A_o - A_t}{A_o} \times 100 \quad (3)$$

Where A_o was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract.

All these tests were performed in duplicate and the graph was plotted with the mean values.

Principle

1, 1- Diphenyl -1- PicrylHydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

$$(DPPH) + (H - A) \rightarrow DPPH - H + (A) \quad (4)$$

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Results and Discussion

Nature and yield of crude extracts of Justicia secunda stem

The extraction of bioactive constituents of *Justiciasecunda* stem using hexane, ethyl acetate, acetone and methanol in order of increasing polarity gave the following results shown in Table 1.

Table 1. Nature and yield of crude extracts of *Justicia secunda*stem

Solvents	Nature/colour of the extract	Yield of extracts
Hexane	Sticky/Dark brown	1.03
Ethyl Acetate	Waxy/Dark brown	1.65
Acetone	Solid/Purple	2.40
Methanol	Solid/Dark brown	4.13

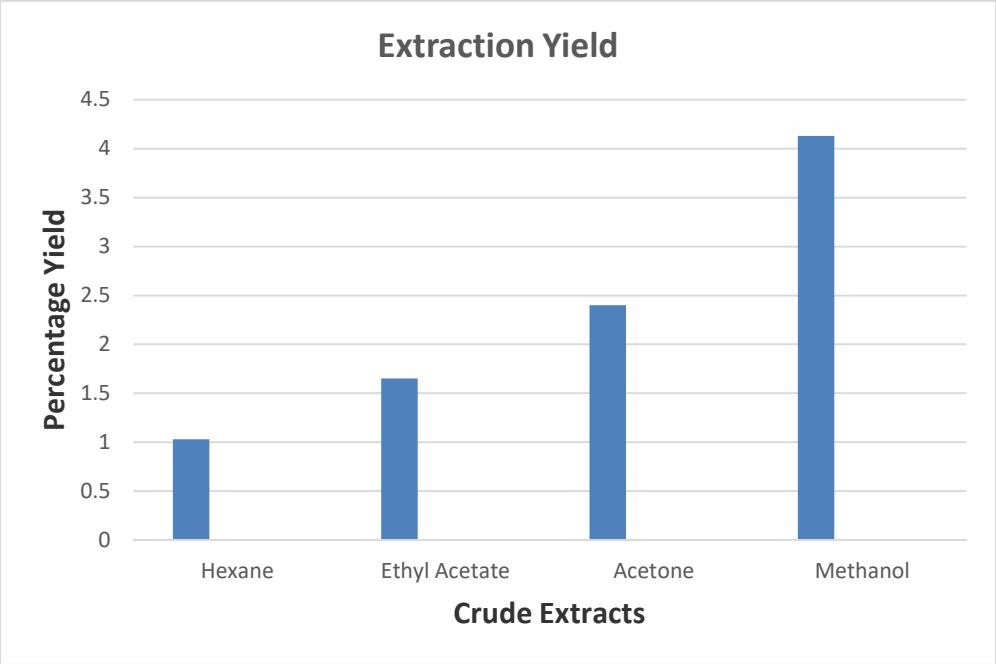

Figure 1. Chart showing the Percentage (%) Yield of the *Justicia secunda* stem Extracts

Phytochemical Screening of Stem Extractsof Justicia Secunda

The hexane, ethyl acetate, acetone and methanol extracts of the stem of *Justicia secunda* were screened for the presence of some phytochemicals such as alkaloids, saponins, flavonoids, tannins, terpenoids, cardiac glycoside, steroids, anthraquinones, phenols and phlobatannins. The following results are presented in Table 2.

Table 2. Phytochemical screening of *Justicia secunda* stem extracts

Phytochemicals	Reagents		Extracts			
			Hexane	Ethyl Acetate	Acetone	Methanol
Alkaloids	a)	Mayer's Reagent	-	+	+	+
	b)	Wagner's Reagent	-	-	+	+
	c)	Dragedroff's reagent	+	+	-	-
	d)	Hager Test	+	+	-	+
Saponins	a)	Froth Test	-	-	-	-
	b)	Foam Test	+	-	+	+
	c)	Emulsion Test	+	+	-	-
Flavonoids			-	++	+	++
Tannins			+	-	+	+
Terponoids			+	+	+	+
Cardiac glycoside	a)	Keller Killani Test	-	+	+	+
	b)	Salkowski Test	+	+	-	+
Steroids			-	+	+	+
Anthraquinones			-	-	+	+
Phenols			+	-	+	-
Phlobatannins			+	+	++	+

Keywords:++ = Abundantltypresent**, + =** present**, - =** absent**.**

Table 3. Quantitative determination of detected phytochemicals of *Justicia secunda*stem extract.

S/No.	DetectedPhytochemicals	Concentration (mg/g)
1.	Alkaloids	0.364
2.	Saponins	0.384
3.	Flavonoids	3.066
4.	Phenols	0.262
5.	Tannins	2.462

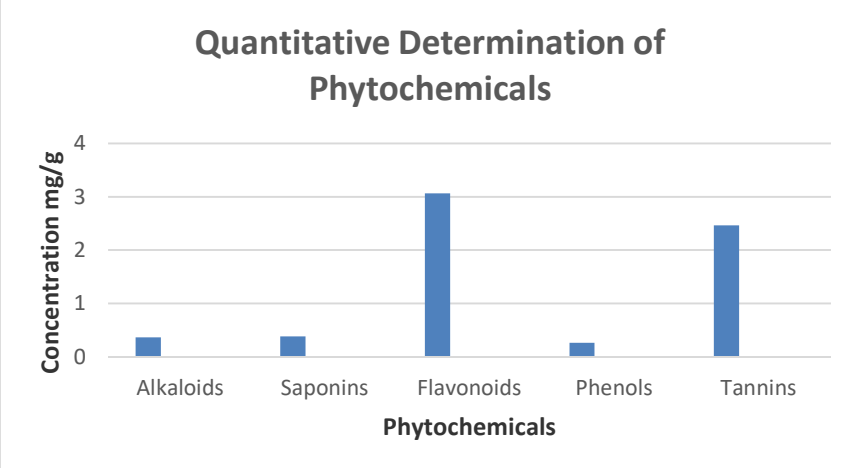

Figure 2. Chart showing the concentration (mg/L) of detected phytochemicals from the stem extracts of *Justicia secunda*.

Table 4. Absorbance of *Justicia secunda* stem extracts and Standard at 517 nm UV-Vis Spectrophotometer

Concentration (mg/mL × 10^{-2})	Absorbance				
	JSSME	JSAE	JSSEE	JSHE	Vitamin C
3.13	0.161	0.151	0.144	0.171	0.133
6.25	0.154	0.148	0.134	0.164	0.124
12.50	0.138	0.132	0.128	0.157	0.108
25.00	0.120	0.120	0.117	0.139	0.085
50.00	0.093	0.099	0.097	0.121	0.044

Blank = 0.185

Table 5. Percentage (%) Inhibition for Standard and *Justicia secunda* stem extracts

Concentration (mg/mL × 10^{-2})	% Inhibition				
	JSSME	JSAE	JSSEE	JSHE	Vitamin C
3.13	12.97	18.38	13.51	7.57	28.11
6.25	16.76	20.54	18.38	11.35	32.97
12.50	25.41	28.65	22.16	15.14	41.62
25.00	35.14	35.68	30.32	24.86	54.05
50.00	49.73	46.22	40.00	34.59	76.22
IC_{50}	**48.04**	**53.71**	**66.03**	**74.62**	**22.76**

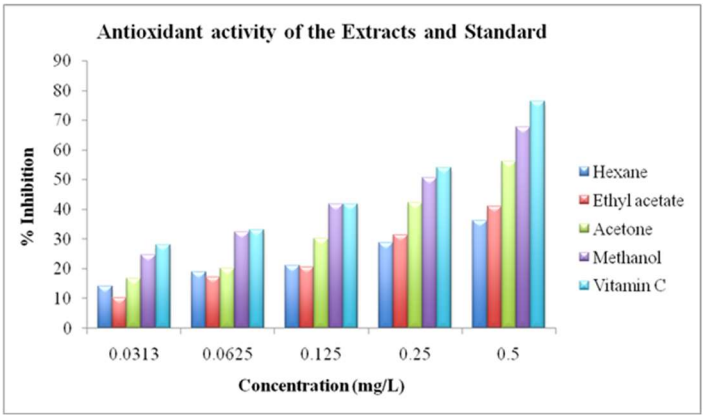

Figure 3. Chart showing the Absorbance of the *Justicia secunda* stem extracts and Standards.

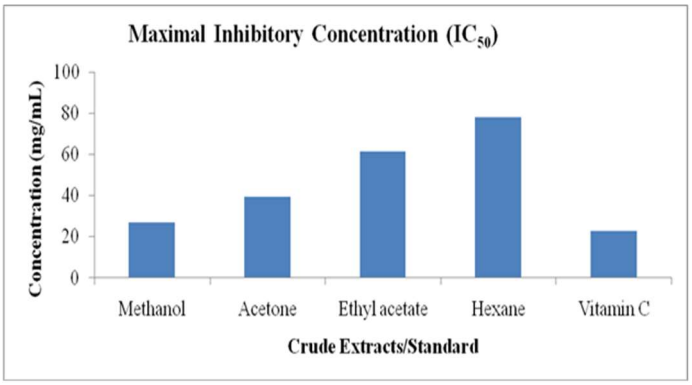

Figure 4. Chart showing the Maximal Inhibitory Concentration of Standard and *Justicia secunda* Stem Crude Extracts.

The result of yield of extract (%), nature and colour of all extracts (Table 1) showed that all extractants used for the serial cold maceration extraction were able to extract some of the *Justicia secunda* stem part but with varied quantity, and physical difference in nature and colour. The extractants were hexane, ethyl acetate, acetone and methanol in order of increasing polarity. The crude extracts of *Justicia secunda* stem showed that methanol had the highest yield of 4.13 %, followed by Acetone 2.40 %, ethyl acetate 1.65 % and the least was hexane with 1.03 %. The differences in the percentage yield could be as a result of the difference in polarity of the solvents, the nature of the constituents involved, variety of bioactive compounds and their differing solubility properties in different solvents. The chemical nature of each of the constituents of the plant parts varies, hence, their solubility in a given solvent. Methanol being a higher polar solvent had higher yield compare to other solvents used in the extraction of the of *Justicia secunda* stem, yet other solvents should have followed in order of increasing polarity as methanol, highest followed by acetone, ethyl acetate and hexane, rather it wasn't like that for all. This could be that the optimal solvent for extraction depends on the particular plant materials and the compounds to be isolated [15].

The results of the preliminary phytochemical screening of *J. secunda* stem revealed the absence of flavonoids in hexane extract but abundantly present in ethyl acetate, methanol extracts and present in acetone extract. Anthraquinones were present only in methanol and acetone extracts and tannins were present in all the extracts except ethyl acetate extracts. Terpenoids were also found present in all the extracts. Phlobatannins were also present in all the extracts and abundantly present only in acetone extract. Phenols were found present only in hexane and acetone extracts. Steroids and Cardiac glycoside were all present in ethyl acetate, acetone and methanol extracts but absence in hexane extract. Saponins and alkaloids were present in all extracts except acetone extract and hexane extract respectively. The variation in the phytochemical composition of the extracts is as a results of the fact that secondary metabolites have different degrees of polarity and hence solubility in different solvents of varying polarity [16].

Justicia secunda can help in healing of wounds and inflamed mucous membrane [17] and decrease bacterial proliferation by blocking key enzymes at microbial metabolism [18] which can be attributed to the presence of tannins. *Justicia secunda* can also be used in treatment of anti-inflammatory diseases because it contains tannins. The presence of terpenoids and cardiac glycoside in the stem extracts of *Justicia secunda* have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic anti-inflammatory and immunomodulatory properties [19]. *J. secunda* is vital in pharmacy because it contains steroidal compounds which are of significance status and interest in pharmaceutical industries due to their relationship with sex hormones [20]. These are well-known to effect the improvement and rheostat of the reproductive tract in humans and molt insects. Other purpose inducing sexual reproduction in aquatic fungi. The quantitative phytochemical screening of the crude extracts of *J. secunda* revealed that the stem was rich in flavonoids 3.066mg/g, followed by tannins 2.462mg/g, saponins 0.384mg/g, alkaloids 0.364mg/g and phenols 0.262mg/g as shown in Table 3. The presence of the detected phytochemicals in the extracts can give medicinal property to the plant part as their presence and nature can make plant to pose therapeutic activity and medicinal value [21]. The highest concentration of flavonoids in the stem of *Justicia secunda,* it's an indication that the stem of *Justicia secunda* possess anti-allergic, anti-inflammatory, anti-microbial and anti-cancer properties. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergies viruses and carcinogens [22].

The crude extracts were assayed for their antioxidant properties by the DPPH radical scavenging method, which evaluates the ability of a sample to scavenge free radicals known to effect biological damages following oxidative stress [23]. The antioxidant activity of the crude extracts

and standard are as shown on Tables 4 & 5 and Figures 3, & 4. Comparing the antioxidants activities of the crude extracts to that of the standard at the various concentrations of 0.0313, 0.0625, 0.125, 0.250, 0.500 mg/mL, the samples showed lower activity than that of the standard. From the absorbance readings of the extracts of *Justicia secunda*stem, it was observed that the absorbancies were decreasing as the concentration increased from 0.313 to 0.500 mg/mL; while the maximal inhibition increased as the concentration increased. The maximal inhibitory concentration (IC_{50}) was obtained from the linear regression analysis for the stem extracts and standard; it was observed the standard had the least IC_{50} value of 22.76 mg/mL indicating highest antioxidant activity, comparing to that of the extracts, methanol extract (48.04 mg/mL) was closely comparable to that of the standard, closely followed by that of acetone extract (53.71 mg/mL), then, ethyl acetate (66.03 mg/mL), and lastly hexane (74.62 mg/mL).

The difference in the scavenging abilities could be, due to the types and concentrations of the phytochemicals each solvent could extract [24]. Phytochemicals which are responsible for scavenging free radical (antioxidants) are mostly polar compounds and are extracted by polar solvents. This result clearly indicates that the stem of *Justicia secunda* possess antioxidant activities, as documented by [25].

Conclusion

It can be concluded from the results, that *Justicia secunda* stem is rich in bioactive compounds which have been reported to possess therapeutic potentials and could be responsible for its antioxidant and antimicrobial effects. The results demonstrated that all of the *Justicia secunda* stem extracts had antioxidant properties; As a result, the plant stem could be a good source of food for humans and animals, a natural antioxidant, and a precursor for the development of antioxidant drugs in the pharmaceutical industry.

Declaration of conflicting interests

The authors declared no potential conflicts of interest

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