PHYTOCHEMICAL ANALYSES AND COMPARATIVE *IN-VITRO* ANTI-OXIDANT STUDIES OF AQUEOUS, ETHANOL AND METHANOL ROOTBARK EXTRACTS OF *Simarouba glauca* DC (Paradise tree)

ABSTRACT

Aims: The study was conducted to determine the presence of selected phytochemicals and in-vitro antioxidant potency of aqueous, ethanol and methanol rootbark extracts of Simarouba glauca. Study Design: True experimental study. Place and Duration of Study: Department of Biochemistry, University of Benin, Benin City, Nigeria, between July and August 2017. **Methodology:** Rootbark of plant was harvested from a private farm at Ubiaja, Esan south east part of Edo State, air dried, pulverized and extracted with water, ethanol or methanol solvents and freeze dried to obtain respective fractions of extracts. Alkaloid, total phenol, tannins and Flavonoid content of rootbark extracts were evaluated by the methods and principles described by Sani et al., Trease and Evans, Sofowora and Santhi and Sengottuvel respectively. DPPH radical scavenging activity, reducing power activity, total antioxidant activity, hydroxyl free radical scavenging activity, ABTS+ radical scavenging activity and nitric oxide radical scavenging activity were evaluated based on the methods and principles described by Kumarasamy et al., Ferreria et al., Benzie and Strain, Wenli et al., Neergheen et al., and Garrat respectively. Results: Alkaloids and flavonoids was observed in aqueous, ethanol and methanol rootbark extracts; flavonoid was however absent in the aqueous fraction. Total phenols and tannin were absent in all fraction. DPPH radical scavenging activity of aqueous, ethanol or methanol fraction extracts recorded no values at 50% inhibition concentration (IC₅₀); although the extracts demonstrated anti-DPPH radical effect at lower inhibitory concentrations, while BHT recorded an IC₅₀ of 18 µg. Reducing power activity of aqueous, ethanol or methanol fraction at IC₅₀ was 11 µg, 10 µg and 11 µg respectively while BHT was 11 µg. Total antioxidant activity of aqueous, ethanol or methanol rootbark extracts at IC₅₀ are 23, 21 and 58 μM FeSO₄ equivalent/μg of Fe²⁺ liberated respectively relative to 388 μM FeSO₄ equivalent/μg of Fe²⁺ liberated by BHT. Hydroxyl free radical activity of aqueous, ethanol and methanol fraction at IC50 was 11 µg, 100 μg and 11 μg respectively while BHT was 11 μg. ABTS⁺ radical scavenging activity of aqueous, ethanol or methanol fraction extracts at IC₅₀ was 29 μ g, 25 μ g and 34 μ g respectively whereas BHT was 21 μ g. Nitric oxide radical scavenging activity of aqueous, ethanol or methanol fraction extracts at IC₅₀ was 14 μ g, 14 μ g and 14 μ g respectively whereas Quercetin was 16 µg. Butylated hydroxyltoluene (BHT) and Quercetin was utilized as standard antioxidant.

Conclusion: The outcome of the research study revealed that the aqueous, methanol and ethanol rootbark extracts of *Simarouba glauca* possesses significant phytochemicals and antioxidant potency, although, the methanol fraction appears to be more effective against investigated radicals.

Keywords: Simarouba glauca; Rootark, phytochemicals; oxidants; radical scavenging properties.

1. INTRODUCTION

Folk medicine have provided substantial information on the traditional health implications of a wide variety of plants. Plants provide naturally occurring phytochemicals as a rich source of antioxidants having free radical scavenging property [1]. Exploration of the plant kingdom for the presence of naturally occurring biologically active phytochemicals that can mitigate; in fact, cure and manage a number of diseases has led to the identification of several plants such as *Simarouba glauca*. *Simarouba* belonging to the family simaroubaceae and widely documented in several pharmacopoeias for their medicinal value [2]. Among the thousands of naturally occurring constituents so far identified in plants and exhibiting a long history of safe use, there are none that pose – or reasonably might be expected to pose a significant risk in human health at low levels of intake when used as flavouring substances.

Studies hitherto conducted on other vegetative parts of this plant has revealed potent availability of phytochemicals and anti-oxidant properties [18], hence the need to explore the health benefits of the rootbark, as studies have shown that the root of plants can accommodate significant amount of active principles of medicinal interest.

ANTIOXIDANTS

Antioxidants are reducing agents and limit oxidative damage to biological structures by passivating free radicals [3]. Antioxidant can be categorized into two main types called **primary** and **secondary antioxidants** where each type is responsible for different mechanisms [4]. Primary antioxidant acts to scavenge free radicals in order to inhibit chain initiation and to break chain propagation by donating hydrogen atoms or electrons that convert them into a more stable product. Secondary antioxidant functions by suppressing formation of radicals and protect against oxidative damage. Besides, secondary antioxidant is also active in binding with metal ions and scavenging oxygen radicals.

1. Botanical Description

Simarouba glauca is a medium sized evergreen tree with tap root system and cylindrical stem. The plants are polygamodioecious with about 5% of the population producing exclusively staminate (male) flowers and 40-50% producing mainly male flowers and a few bisexual (andromonoecious) while the remaining 40-50% produces only the pistillate (female) flowers.

Botanical name: Simarouba glauca DC

Family: Simaroubaceae

Genus: Simarouba

Species: glauca

Synonyms: Quassia Simarouba,, Simarouba

medicinalis

Common Names: aceituno, paradise tree, negrito,

Dysentery bark, palo blanco

Technical Data Report for Simarouba glauca (2002)

2. MATERIALS AND METHODS

2.1 Plant Materials

The rootbark of *Simarouba glauca* was harvested from a private farm located at Ubiaja, Esan -South East Local Government Area of Edo State. Samples were transported to Department of Biochemistry, air dried for approximately 29 days and pulverised at the Department of Pharmacognosy Laboratory, in the University of Benin.

2.2 Preparation of Plant Extracts

A 400 g of the rootbark of *Simarouba glauca* was weighed and submerged in 5 litres of water, ethanol or methanol for 24 hours, after which the mixture was sieved off to obtain the filtrate. The filtrate was decanted into a clean sterile container and the residue was subsequently submerged in a 3 L of water, ethanol or methanol of 99% purity for another 24 hours. The same procedure as earlier stated was carried out to obtain the filtrate. The filtrate was transported to the Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko, Ondo State; freezedried to obtain pure extracts of water, methanol and ethanol. These extracts were stored in sterile bottles and kept in the refrigerator at -4°C until required for analysis.

2.3 CHEMICALS

2.3.1 Phytochemistry

Distilled water, Hagers reagent, 10% Ferric chloride, 0.1% Ferric chloride, Lead acetate solution, Sulphuric acid.

2.3.2 Antioxidants

Methanol, 2,2-Diphenyl-1-Picrylhydrazyl(DPPH), Butylated Hydroxyltoluene (BHT), Phosphate buffer (pH6.6), Disodium hydrogen phosphate (Na₂HPO₄), Sodium dihydrogenphosphate (NaH₂PO₄), Potassium Ferricyanide, Trichloroacetic acid (TCA), Ferric Chloride (FeCl₃), Distilled water, Acetate buffer (pH 3.6), Sodium acetate, Glacial acetic acid, Hydrochloric acid, Ferrous sulphate, 2, 4, 6- tripyridyl-triazine (TPTZ), FRAP reagent, 1, 10-Phenanthroline, Phosphate buffer (pH 7.4), Hydrogen peroxide, 2,2- Azinobis (3- ethylbenzothiazoline-6-Sulphonic acid) (ABTS), Potassium persulphate, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Sodium nitroprussside, Phosphate buffer Saline (pH 7.4), Sodium Chloride, Potassium Chloride, Potassium dihydrogenphosphate (KH₂PO₄), Sodium Hydroxide, Sulphanilic acid, Naphthylethylenediaminehydrochloride, Quercetin, Distilled H₂O, Ethanol and Methanol (99% purity).

2.4 Identification of Alkaloids

Alkaloid of rootbark was determined in accordance with the principle described by Sani [5]. To 2ml of the root extract of *Simarouba glauca*, 2ml of 10% hydrochloric acid was added. To the acidic medium, 1ml Hager's reagent (saturated picric acid solution) was added. Presence of Alkaloid is confirmed by formation of yellow coloured precipitate.

2.5 Identification of Phenols

The qualitative determination of total phenol was conducted by the method described by Trease and Evans [6]. 2ml of root extract of *Simarouba glauca* was mixed with few drops of 10% Ferric chloride solution. The formation of green-blue or violet or blue-black colouration indicates the presence of phenolic compounds.

2.6 Identification of Tannins

The identification of tannin was determined by the method described by Sofowora [7]. To 2ml of root extract of *Simarouba glauca*, 5 drops of 0.1% Ferric chloride was added. The formation of a brownish green or blue-black colouration indicates a positive presence of tannins.

2.7 Identification of Flavonoids

The total flavonoid in aqueous, ethanol and methanol rootbark extracts was determined by the methods described by Santhi and Sengottuvel [8].

Lead acetate test: To 2ml of root extract of Simarouba glauca, few drops of Lead acetate solution was added. Formation of yellow coloured precipitate indicates the presence of flavonoids.

Sulphuric acid (H₂SO₄) method test: To 2ml of root extract of Simarouba glauca, few drops of H₂SO₄ solution was added. Formation of orange coloured precipitate indicates the presence of flavonoids.

2.8 2, 2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) RADICAL SCAVENGING ASSAY

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) is a stable free radical with red color (absorbed at 517nm). If free radicals have been scavenged, DPPH will generate its colour to yellow. This assay uses this character to show herbs free radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is widely used in plant biochemistry to

evaluate the properties of plant constituents for scavenging free radicals. Butylated Hydroxyl Toluene (BHT) was used as the positive reference standard. It behaves as a synthetic analog of vitamin E, primarily acting as a terminating agent that suppresses autoxidation. BHT is known as a synthetic antioxidant [9]. The DPPH test showed the ability of the test compound to act as a free radical scavenger. This assay is based on the ability of DPPH to decolourize in the presence of an antioxidant. A serial concentration gradient of 0.01- 0.12 mg/ml which is equivalent to 10 μ g/ml, 20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml, 100 μ g/ml and 120 μ g/ml was prepared from a stock of 1mg/ml. A solution of 0.1 mM DPPH in methanol was prepared. 1.0 ml of the solution was mixed with 3.0 ml of extracts in methanol of the concentration range of the extract. The mixture was vortexed thoroughly and kept in the dark at room temperature for 30 minutes. The absorbance is read spectrophotometrically at 517 nm. The result of the radical scavenging activity of DPPH was expressed as percentage inhibition and inhibition concentration at 50 % (percent) (IC₅₀), i.e. the concentration of extract required to stop the radical chain reaction of DPPH by 50 % (IC₅₀) under the experimental conditions [10].

DPPH radical scavenging activity was calculated using the following equation:

Percentage (%) inhibition = $[(A_0 - A_1) / (A_0)] \times 100$

2.9 REDUCING POWER ASSAY

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron method, in the reduction of Fe³⁺ (CN⁻) 6 to Fe²⁺ (CN⁻) 6 [11]. A 1 ml of concentration gradient of extracts (0.02 - 0.12 mg/ml) in methanol is mixed with 2.5 ml of 0.2 M phosphate buffer pH 6.6, 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. Thereafter, 2.5 ml of 10% trichloroacetic acid was added to stop the reaction. 2.5 ml of distilled water and 0.1% FeCl₃ were added and the absorbance was measured at 700 nm. Higher absorbance values indicated higher reducing power. Butylated hydroxyl toluene served as positive control.

% Radical scavenging activity RSA = $[(A_0 - A_1) / (A_0)] \times 100$

2.10 TOTAL ANTIOXIDANT ASSAY (FRAP)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain [12]. It is based on the ability of the sample extracts to reduce Ferric tripyridyltriazine (Fe (III)-TPTZ) complex to Ferrous tripyridyltriazine (Fe (II)-TPTZ) at low pH. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593 nm. The reaction is nonspecific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture. The FRAP reagent was generated by mixing 25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM 2,4,6- tripyridyl triazine (TPTZ) solution in 40 mM HCl and 2.5 ml of 20 mM Ferric chloride (FeCl $_3$.6H $_2$ 0) solution. Samples at different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) was then added to 1.5 ml of freshly prepared FRAP solution and 7.5 ml of distilled water. The absorbance was read at 593 nm. Fresh solutions of FeSO $_4$ were used as standard.

% Radical scavenging activity RSA= $[(A_0 - A_1) / (A_0)] \times 100$

2.11 HYDROXYL FREE RADICAL SCAVENGING ASSAY

The hydroxyl free radical scavenging activity was conducted according to the method described by [13]. To 1 ml of the extracts with different concentrations (0.02-0.12 mg/ml) was added 1ml of 0.75 mM 1,10- phenanthroline, 1.5

ml of 0.75 mM FeSO₄ , 3.8 ml of 0.2 M phosphate buffer pH 7.4, 1 ml of 0.01% H₂O₂ and 2.6 ml of distilled water. The absorbance was read at 536 nm.

% Radical scavenging activity RSA= $[(A_0 - A_1) / (A_0)] \times 100$

2.12 ABTS⁺ RADICAL SCAVENGING ACTIVITY

This assay was conducted with an improved 2,2-azinobis-(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) based on the principle of decolourization [14] as described by Neergheen [15]. The ABTS⁺ radical was generated by a reaction between ABTS (0.5 mM) and 1 mM potassium persulfate in 0.1 M phosphate buffer. To 0.5 ml of the extract with concentration range of 0.04-0.14 mg/ml was added 3 ml of ABTS (2,2'- Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt). Absorbance was read at 734 nm.

% Radical scavenging activity RSA= $[(A_0 - A_1) / (A_0)] \times 100$.

2.13 NITRIC OXIDE RADICAL SCAVENGING ASSAY

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO*), which interacts with oxygen to produce nitrite ions. Scavengers of NO* compete with oxygen, leading to reduced production of NO* and a pink coloured chromophore is formed [16]. A 2ml of 10 mM Sodium nitroprusside was added to 0.5 ml of the extract with concentration range of 0.02-0.10 mg/ml. 0.5 ml of phosphate buffer saline pH 7.4 was added to the reaction mixture. The mixture was incubated at 25°C for 150 minutes. After incubation, 0.5 ml is pipette from the tubes into a new set of tubes. To the new set of tubes, 1 ml of 0.33 % Sulphanilic acid was added and allowed to stand for 5 minutes for complete diazotization. Thereafter, 1ml of 0.1% Naphthylethylenediamine dihydrochloride was added and mixed thoroughly and allowed to stand for 30 minutes. Absorbance was read at 540 nm.

% RSA = 1- <u>Absorbance of sample</u> × 100 Absorbance of control

3. RESULTS AND DISCUSSION

3.1 Alkaloids

The results of alkaloids detected in aqueous, ethanol and methanol rootbark extracts of *S. glauca* are shown in table 1. The study showed that aqueous rootbark extract contained more alkaloid content compared to ethanol and methanol rootbark extract. Alkaloids, an amazing group of phytochemical, isolated from medicinal plants, have a varied spectrum of effects such as analgesics to addictive [17] and those of edible mushrooms have demonstrated anti-hypertensive potentials [22]. Alkaloids play major role as anticancer agents by inhibiting the enzyme topoisomerase which is involved in DNA replication, inducing apoptosis and expression of p53 gene [17]. Alkaloids have potent anticancer activity against various cancers [17]. The strength of the yellow precipitate observed of the aqueous extracts revealed the strong presence of alkaloids.

3.2 Flavonoids

The results of flavonoids detected in aqueous root extract, methanol and ethanol root extract of *S. glauca* are shown in table 1. The study revealed that flavonoid was strongest in methanol rootbark extract compared with aqueous or ethanol rootbark extract. Flavonoid promotes antioxidant activity that helps to maintain normal tissue growth.

3.3 Phenol

Phenol compounds was not detected in the extracts of S. glauca.

3.4 Tannin

The study showed that tannin was neither present in the aqueous, ethanol nor methanol rootbark extracts of *S. glauca*.

Table 1. Phytochemicals constituents present in extracts of Simarouba glauca.

Phytochemical	Aqueous Extract	Ethanol Extract	Methanol extract
Alkaloids	+++	+	++
Flavonoids	-	+	++
Phenols	-	-	-
Tannins	-	-	-

3.5 2,2-DIPHENYL-1-PICRYLHYDRAZYL SCAVENGING FREE RADICAL

In DPPH radical scavenging activity, the aqueous extract demonstrated maximum scavenging activity of 10.64 % at 60 μg concentration of root bark extract. The ethanol extract indicated maximum activity of 16.45 % at 40 μg concentration of extract whereas BHT showed maximum activity of 78.80 % at 60 μg concentration. The methanol extract indicated maximum activity of 26.25 % at 10 μg concentration of extract. The IC₅₀ indicates the concentration at which 50% of the test substance is significantly affected. Standard BHT was found to have an IC₅₀ value of 18 μg. The aqueous, methanol and ethanol extract indicated no IC₅₀ value. A higher IC₅₀ value indicates lesser free radical scavenging activity. Higher percentage (%) inhibition value indicates higher potent activity. From the result, it can be observed that aqueous, methanol or ethanol rootbark extracts were unable to scavenge free radicals at 50% inhibitory concentration.

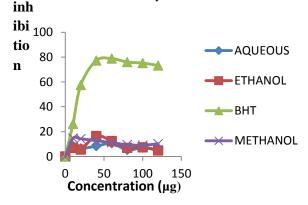


Fig 1. DPPH radical scavenging activity of S. glauca Rootbark extracts and butylated hydroxytoluene (BHT)

3.6 REDUCING POWER ACTIVITY

Reducing power activity of aqueous, ethanol or methanol root extracts of *Simarouba glauca* were compared to BHT. It was observed that control BHT had a concentration of 11.0 μ g at 50% inhibition. The IC₅₀ values of aqueous, ethanol and methanol rootbark extracts where observed to be 11.0, 10.0 and 10.5 μ g respectively. The aqueous rootbark extract exhibited maximum reducing capacity of 96.58% at 100 μ g. Ethanol rootbark extract exhibited

maximum reducing capacity of 97.53% at 20 μ g, while the methanol rootbark extract exhibited maximum reducing capacity of 97.45 % at 40 μ g.

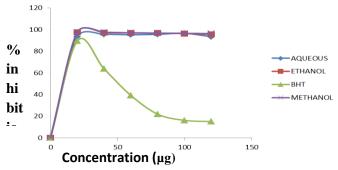


Fig.2. Reducing power activity of S. glauca rootbark extracts and Butylated hydroxytoluene (BHT)

3.7 TOTAL ANTIOXIDANT ACTIVITY (FRAP) ASSAY

S. glauca rootbark extracts reducing potential was estimated by its ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. Reducing activity increases as concentration increases. A standard curve of FeSO₄.7H₂O (10-100 μ M) was plotted against concentration (μ g) wherein the amount of Fe (II) liberated by extract's reducing activities including BHT was extrapolated. At 50% inhibition concentration, aqueous rootbark extract liberated 22.84 μ M FeSO₄ equivalent/ μ g of Fe²⁺, ethanol rootbark liberated 20.87 μ M FeSO₄ equivalent/ μ g of Fe²⁺ while methanol rootbark extract liberated 58.03 μ M FeSO₄ equivalent/ μ g of Fe²⁺ vis-à-vis 388.23 μ M FeSO₄ equivalent/ μ g of Fe²⁺ liberated by BHT. This indicates that the methanol extract has a greater ability to reduce TPTZ-Fe³⁺ to TPTZ-Fe²⁺.

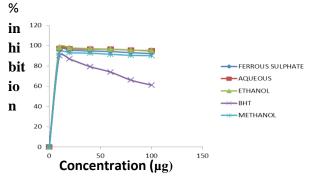


Fig.3. FRAP of S. glauca rootbark extracts and Standard BHT vis-a-vis Fe (II) liberated.

3.8 HYDROXYL FREE RADICAL SCAVENGING ACTIVITY

At 20 μ g, it was observed that the aqueous extract, methanol extract and standard BHT had a remarkable scavenging activity of 93.83%, 91.63 % and 93.49% respectively and are present in figure 4 and table 2. The IC₅₀ value of ethanol rootbark extract was observed to be 100 μ g compared to aqueous or methanol rootbark extracts with IC₅₀ values of 11 and 11 μ g respectively. BHT also recorded 11 μ g IC₅₀.

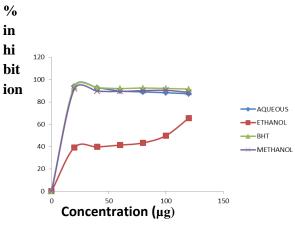


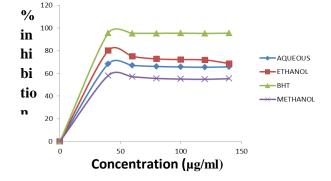
Fig 4. Hydroxyl free radical scavenging activity of *S. glauca* rootbark extracts and Butylated hydroxytoluene (BHT).

3.9 ABTS+ RADICAL SCAVENGING ACTIVITY

The capacity of aqueous, ethanol or methanol rootbark extracts of S. glauca and standard BHT to reduce the ABTS⁺ radical which is generated by a reaction between ABTS⁺ and potassium persulphate in phosphate buffer are shown in figure 5 and table 2 respectively. The scavenging capacity initially increases and then decreases with increase in concentration. The IC_{50} values of aqueous, ethanol and methanol root extract was observed to be 29.0, 25.0 and 34.0 μ g respectively, whereas IC_{50} value for BHT recorded 21 μ g. Ethanol rootbark extract demonstrated a better inhibition effect of 25 μ g at IC_{50} compared to aqueous and methanol rootbark extracts with 29 and 34 μ g at IC_{50} respectively.

Table 2. Percentage (%) inhibition concentration of Aqueous, ethanol and Methanol rootbark extracts of Simarouba glauca against radicals and standard.

Antioxidant/	Inhibition concentration (IC ₅₀)					
radical	Aqueous	Methanol	Ethanol	BHT	Quercetin	
	rootbark extract	rootbark extract	rootbark extract			
DPPH (µg)	NIL	NIL	NIL	17.90	-	
Reducing power (µg)	11.00	10.50	10.00	11.00	-	
$FRAP(\mu g)$	22.84	58.03	20.87	388.23	-	
Hydroxyl radical (µg)	11.00	11.00	100.00	11.00	-	
ABTS ⁺ radical (µg)	29.00	34.00	25.00	21.00	-	
Nitric oxide (µg)	14.00	14.00	14.00	-	15.90	
Nitric oxide (µg)	14.00	14.00	14.00	-	15.90	



3.10 NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

The aqueous, ethanol or methanol rootbark extracts and Quercetin being the standard antioxidant demonstrated % significant nitric oxide Radical scavenging activity with IC₅₀ values of 14.0, 14.0, 14.0 and 16.0 μg respectively as inhihown in figure 6 and table 2. All extracts remarkably displayed equipotent inhibition against nitric oxide radicals bition in fact demonstrated a better anti-nitric oxide radical activity compared to Quercetin.

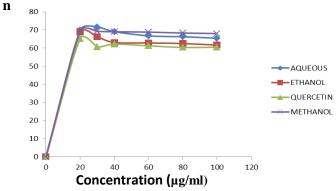


Fig 6. Nitric oxide radical scavenging capability of S. glauca rootbark extracts and Quercetin

4. CONCLUSION

The results obtained from the study is evident that *S. glauca* possesses strong antioxidant potency and relevant phytochemicals capable of scavenging free radicals harmful to cells; also maintain the integrity of the cells. Such properties of plants have been implicated in the management and treatment of a number of diseases and conditions too numerous to mention. Therefore, the findings in this study serves a useful information on further studies of *Simarouba glauca* with respect to pharmacological evaluation and potentials in prevention, management and treatment of related conditions and diseases.

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