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Exploring the phytochemistry and *in vitro* antioxidant potentials of hot water extract of *Ocimum gratissimum* (Scent Leaf)

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Abstract
The aim of this study is to investigate the activities of phytochemicals and *in vitro* removal of free radicals from hot water extracts of *Ocimum gratissimum*. Spectrophotometric assessments were used to determine the total phenol, the total flavonoids, the total alkaloids, the total tannins, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitrogen oxide (NO), total antioxidant capacity (TOAC) and the reduction power (RP). Qualitative phytochemical examinations confirmed the presence of phytochemical substances such as terpene, phenol and tannin. In quantitative analysis, the results are as follows: total phenol (8.47±1.33 mg/gGAE) tannin (7.84±0.13 mg/gTAE) alkaloids (2.70±0.05 mg/gATE). The inhibitory effect of the nitrous oxide radicals of the *Ocimum gratissimum* extract and its overall antioxidant capacity increased significantly ($p<0.05$) with increasing concentrations, and the inhibitory effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of the extract and its reduced resistance decreased significantly ($p<0.05$) depending on the dose. The results of this study show that hot water extract of *Ocimum gratissimum* has antioxidant properties and that extraction protocols have improved these properties.

Keywords: Phytochemicals, antioxidants, *Ocimum gratissimum*, aqueous extracts

1. Introduction
Plants have continuously been investigated for their medicinal properties because they contain abundant amounts of polyphenolic compounds and other related plant pigments which make up the therapeutic properties of plants known as phytochemicals. They are also known as bioactive compounds in plants (Asiwe *et al*., 2023a) [3]. These phytochemicals include; tannins, saponins, alkaloids, flavonoids, polyphenols, etc (Ezeokeke *et al*., 2015, Asiwe *et al*., 2023b) [11, 4]. These compounds are responsible for the medicinal attributes of plants and have lead to the use of plants in clinical trials for chemotherapy and prophylaxis; this is not only as a result of the abundance of these phytonutrients in plants but also due to their minimal side effects. They have been shown to be abundant-sources of exogenous antioxidants (Enenebeaku *et al*., 2022) [9-10] that inhibit the reactions of free radicals.
The energy metabolic pathway has side reactions, sometimes causing cell damage that leads to cell membrane loss, stability, and apoptosis. Free radicals the species of reactive oxygen (ROS) and the species of reactive nitrogen (RNS)–produced by these metabolic side reactions and external sources are known to cause degenerative or pathological diseases such as aging, cancer (Alisi *et al*., 2018) [1], diabetes, etc. Paduraneanuet *et al*., 2019) cause disruption of normal process of metabolism.
The basic building blocks of proteins, lipids, nucleic acids and carbonhydrates (Rahmen *et al*., 2015) [25] are damaged by these radicals leading to the production of compounds that are detrimental to living cells, and they serve as oxidative stress indicators (Rusak *et al*., 2018) [27]. Since most diseases are said to be caused by oxidative stress, modern methods of providing a cure to these diseases should be considered from the view of forestalling reactions of oxidative stress. This is probably accomplished through free radical scavenging activities of medicinal plants (Ramu & Mohan., 2019) [26]. But endogenous antioxidants that can perform the scavenging of free radicals can deplete with time, so a supplementary agent is required in the form of exogenous antioxidants that are found in most plants. Synthetic antioxidants are not being used because of the toxic and mutagenic effects of most of their components.
Ocimum gratissimum is an aromatic perennial herb that is found throughout the tropics and subtropics and its variants are mostly located in tropical Africa and Asia (Mann, 2012) [17]. It belongs to the family *Lamiaceae*.

In folklore medicine, the leaves of this plant are used as an all-purpose tonic and anti-diarrhea agent, and for the management of conjunctivitis by passing the fluid directly into the eye, skin infections, bronchitis, headache, fever and other uses. Its nutritional importance lies on its usefulness as a seasoning because of its aromatic flavor (Duru *et al*., 2021) [8]. It is also used in fragrance, dental and oral products (Da Silva Gundel *et al*., 2018). The present study therefore explored the phytochemical contents and *in vitro* antioxidant activities of hot water extract of *Ocimum gratissimum.*

Fig 1: Scent leaf (*Ocimum gratissimum*)

2. Methods

2.1 Collection and authentication of plant material

The leaves of *Ocimum gratissimum* were harvested from a fallow farmland in Abraka community of Delta State in February, 2023 and were identified and validated by Dr. Harrison A. Erhenhi of Botany Department, Delta State University, Abraka, Nigeria.

2.2 Extract preparation

Five hundred grams (500g) of *Ocimum gratissimum* matured leaves were freshly harvested, cleaned and washed with running water. The leaves were then transferred into a clean pot containing 2000ml (2 litres) of tap water and boiled for 15mins using mild heat regulation on a kerosene stove. The extract was allowed to cool and filtered with muslin cloth, the filtrate was filtered again using Whatman No. 1 filter paper. Filtrate was then stored in a refrigerator at 4 ^{o}C before being used for the study.

2.3 Qualitative phytochemical determination

Phytochemicals in the extract were determined using standard of Borokini and Omotayo (2012) [6], and Njoku and Obi (2009) [19] to ascertain the presence of various chemical components.

2.3.1 Test for saponins

Frothing test was used, to 1g of sample, 30ml of distilled water was added and the mixture was strongly shaken. Samples were then observed for froth formation.

2.3.2 Test for phlobatanin

0.2g of sample was dissolved in 10ml of distilled water and filtered, 2ml of the filtrate was boiled with 2ml of 2% hydrochloric acid solution. Presence of red precipitate showed the presence of phlobatanin.

2.3.3 Test for cardiac glycosides

The Keller-Killani test was used, 5ml of the sample and 2ml of glacial acetic acid was added to a test tube, one drop of 2% ferric chloride solution was added, 1ml of conc. H_2SO_4 was added. A brown interface, violet ring colour and a greenish ring at the lower part indicated presence of cardiac glycosides.

2.3.4 Test for flavonoids

Shinoda tests were used. To the test tube, which contains 0.5 ml of samples, add10 drops of concentrated HCl and a small piece of magnesium, and boil the solution for 5 minutes, reddish-pink colour indicates that flavonoids are present.

2.3.5 Test for tannins

The Ferric chloride test was used to determine this, in 0.2 ml of the sample extract a few drops of 5 percent water-soluble ferric chloride solution was added. A bluish black color disappears when a few milliters of diluted sulfuric acid is added, followed by yellow brown precipitation, indicating the presence of tannin.

2.3.6 Test for phenol

Ferric chloride testing was used. In 1.0 ml of sample extract,2 ml of distilled water, and a small drop of 10% of water-rich iron chloride solution were added. The formation of blue dyes indicates the presence of phenols.

2.3.7 Test for steroids

To 2 ml of acetic anhydride,0.5 g of sample extract, 2 ml of sulphur acid were added. The blue color indicates the presence of steroids

2.3.8 Test for terpenes/terpenoids

Salkowski test was used to mix 5ml of samples with 2ml of chloroform and 3ml of concentrated sulfuric acid to form layers. Redish brown colouration shows the presence of terpenoids/terpenoids.

2.4 Quantitative phytochemical analysis

2.4.1 Estimation of total phenols and total tannin (Pandey *et al*., 2015) [22]

The phenol compounds are oxidized to phenolates by the Folin-Ciocalteau's reagent at alkaline pH in a saturated sodium solution.

2.4.2 Procedure

Extracts from the leaves of *Ocimum gratissimum* was mixed with 1.0 ml of Follin Ciolcalteau phenol reagent, and after 3 minutes, 1.0 ml of saturated Na_2CO_3 was added to the mixture, and the amount reached 10 ml by adding distilled water. The reaction mixture is stored for 90 minutes in the dark and then read at an absorbance of 725 nm. The calibration curves of the phenol and the tannic acid (20-100/gml) were designed, respectively. The results are expressed as equivalent to mg gallic acid (GAE), equivalent to mg g extract, and equivalent to mg tannic acid (TAE), depending on the respective results.

2.4.3 Evaluation of total flavonoids (Jia *et al*., 1999) [14]

Principle

The sample was mixed with a reagent containing aluminum chloride and sodium nitrate. A pink flavonoid aluminum

complex was formed in the alkaline medium. The color intensity was read at 510 nm.

2.4.4 Procedure
A sample tube containing 1.25 ml of distilled water and 0.075 ml of 5% of $NaNO_2$ solution was added to 0.5 ml of the hot water leaf extract of *Ocimum gratissimum*. After 5 minutes of storage, 0.15 ml of 10% $AlCl_3$ was added, and after 6 minutes, 0.5 ml of 1.0 M NaOH was added, and the mixture was diluted with 0.275 ml of distilled water. The absorption was immediately measured at 510 nm. Catechin was the standard (20-100 g/ml). The total content of flavonoids was expressed in milligrams of catechin (CAE) equivalents per g extract.

2.4.5 Evaluation of total alkaloids (Shamsa *et al*., 2008) [12]
Principle
This method is based on the reaction between the alkaloid and the green bromide (BCG), and forms a yellow complex that can be easily be extracted from chloroforms at pH 4.7.

2.4.6 Procedure
To 1ml hot water leaf extract of *Ocimum gratissimum*, a 5ml pH 4.7 phosphate buffer was added and a 5ml BCG solution, the mixture was shaken after adding 4ml chlorine. The extract was collected in a 10 ml volume bottle and diluted to adjust the volume with chloroform. The absorption of the chlorine complex was measured at 470 nm compared to the previous blanks without extract. Atropine was used as a standard material and compared to the equivalents of Atropine (40–120g/ml).

2.5 *In-vitro* antioxidants activities
Determination of antioxidant activity and free radical scavenging of leaf extracts of *Ocimum gratissimum* 1, 1-diphenyl -2-picryl hydrazyl (DPPH) assay (Lim and Quah (2007)
Principle
DPPH (1-diphenyl-2-picryl hydrazyl) radicals are scavenged by antioxidants through the donation of protons that form reduced DPPH. The change in colour from purple to yellow can be quantified by the reduction of the absorbance at 517 nm.

Procedure
0.2 ml of different concentrations of the extract (3 to 21% w/v) are added to 2 ml of DPPH solution (0.3 mM). After 30 minutes of incubation in the dark, the absorption rate was 517 nm. The percentage of DPPH radical elimination inhibition is calculated using the following equation% inhibition of DPPH radical = $([A_0–A_1]/A_0) \times 100$, A_0 is the control absorbency (black, without extraction) and A_1 is the absorption rate in the presence of extraction.

Nitric oxide (NO) free radical scavenging activity (Marcocci *et al.*, 1994) [18]
Principle
Sodium nitroprusside is spontaneously produced in aqueous solution at physiological pH generate nitric oxide, but interacts with oxygen to produce nitrate ions at 540nm which is estimated spectrophotometrically at 540 nm.

Procedure
Two milliliters of 10mM sodium nitrate dissolved in 10mM phosphate buffer sodium (pH 7.4), was mixed with 0.5ml different concentrations of hot water leaf extract of *Ocimum gratissimum*, 3–21% w/v). The mixture was then incubated at 25 °C. After 150 minutes of incubation, 0.5 ml of the incubated solution was removed and mixed with 0.5 ml of the Griess reagent [(1.0 ml of sulfuric acid reagent with 0.13% acetic acid with 1 ml of naphthylethylenediamine dichloride (0.1% w/v) at ambient temperature for 5 minutes. The mixture was then cooled at ambient temperature for 30 min and measured by a 546nm absorbance on the blank. The percentage inhibition of nitric oxide radical scavenging is calculated by using the following equations:

% inhibition of NO radical = $([A_0 – A_1]/ A_0) \times 100$

Where A_0 = absorbance of the control (blank, without extract)
A_1 = absorbance in the presence of the extract.

Reducing power assay (RP) (Sofidiya *et al*., 2006) [28]
Principle
The reduction power was measured by direct electron donation for $Fe^{3+}(CN^-)_6$ reduction to $Fe^{2+}(CN^-)_6$, and after the reduction reaction, the free Fe^{3+} ions were added, forming a solid blue complex $Fe_4{}^{3+}[Fe^{2+}(CN^-)_6]^3$ and quantified by 700nm spectrophotometer absorption.

Procedure
Determination of total antioxidant capacity (Prieto *et al*., 1999) [24]

Principle
The test is based on removing the Mo(VI)-Mo(V) complex and then forming the Green Phosphate/Mo(V) complex at acid pH. The solution absorbs at 695 nm.

Procedure
The solution of 0.0 ml of the reaction solution (0.6 mL of sulfuric acid, 28 mL of sodium phosphate, and 4 mL of ammonia membrane) was taken into a screw-capped tube and added 0.1 ml of the extract of hot water leaf, *Ocimum gratissimum*. The tubes were closed and incubated for 90 minutes in a heat block at 95 °C. After cooling to ambient temperature, the absorption of water solution from each tube was measured at 695 nm in blanks. Gallic acid (20-100 /gml) was used as a standard and the total antioxidant capacity is expressed as a GAE equivalent.

Statistical analysis
All data were statistically analyzed. The values were reported as average standard deviation, and one-way ANOVA was used to verify differences between treatment groups. The results were considered significant with a p-value below 0.05, at a 95% confidence level ($p<0.05$). The Turkey HSD post-Hoc tool was used for basis of statistical comparison.

3. Results and Discussion
3. Results
Qualitative Phytochemical screening of hot water leaf extracts of *Ocimum gratissimum:* Results of qualitative phytochemical screening of hot aqueous leaf extract of *Ocimum gratissimum s*howed that the plant contains alkaloids, flavoids, phenols, tannins, terpenes. While the quantitative phytochemical screening of the same extract showed high concentrations of phenol, flavonoid, tannin, and alkaloid.

Table 1: Qualitative phytochemical Screening of hot aqueous leaf extracts of *Ocimum gratissimum*

Phytochemicals	Aqueous extract
Saponin	-
Tannin	+
Terpenes	+
Steroid	-
Alkaloid	+
Phlobatanin	-
Cardiac glycoside	-
Flavonoid	+
Phenol	+

Key + = Present, - = Absent

Quantitative determination of phytochemical composition of hot water leaf extract of *Ocimum gratissimum*

Results of quantitative phytochemical analysis of hot water leaf extract of *Ocimum gratissimum* are presented in table 2.0. Concentration of phenol was highest (8.47±1.33mg/gGAE), then tannin (7.84±0.13mg/gTAE) and the lowest was alkaloid (2.70±0.05 mg/gATE).

Table 2: Quantitative phytochemical analysis of hot aqueous leaf extract of *Ocimum gratissimum*

Phytochemicals	Aqueous extract
Phenols (mg/gGAE)	8.47±1.33
Flavonoid (mg/gCAE)	5.82±1.01
Tannin (mg/gTAE)	7.84±0.13
Alkaloid (mg/gATE)	2.70±0.05

Figures are means ± standard deviations of three determinations. GAE = Gallic acid equivalent, CAE = Catechin equivalent, ATE = Atropin equivalent, GluE = Glucose equivalent. TAE = Tannic acid equivalent.

In-vitro free radical scavenging activities of hot aqueous leaf extract of *Ocimum gratissimum*

Results of the antioxidant activity of the hot water leaf extract of *Ocimum gratissimum* are shown in Table 3.0. The results showed that the activity of the extract against nitric oxide and the total antioxidant capacity ($p<0.05$) increased considerably as concentrations increased. The reduction of 1,1-diphenyl-2-picrylhydrazyl and reducing power ($p<0.05$) was significantly reduced in dose-dependent manner.

Table 3: *In-vitro* antioxidant activity of hot aqueous leaf extract of *Ocimum gratissimum*

Conc. (w/v%)	% Inhibition		700nm	695nm
	DPPH	NO	RP	TOAC
3	88.8±0.63[a]	22.7±0.78[a]	1.67±0.11[a]	1.26±0.04[a]
6	82.3±0.14[b]	28.0±0.78[b]	1.65±0.01[a]	1.85±0.18[b]
9	82.1±0.04[b]	35.4±1.74[c]	1.54±0.05[b]	2.57±0.02[c]
12	81.6±0.21[b]	45.6±1.74[d]	1.35±0.02[c]	2.65±0.03[d]
15	79.7±0.43[c]	52.4±0.97[e]	1.25±0.03[d]	3.26±0.22[e]
18	79.6±0.33[c]	59.3±0.66[f]	1.41±0.07[e]	3.76±0.10[f]
21	79.5±0.35[c]	67.6±2.14[g]	0.68±0.01[f]	4.29±0.11[g]

Figures are presented as mean ± standard deviations of three determinations. Figures not sharing same superscript on the same column differ significantly ($p<0.05$). DPPH = 1,1 diphenyl-2-picryl hydrazyl, NO = Nitric oxide, RP = Reducing power, TOAC = Total antioxidant capacity.

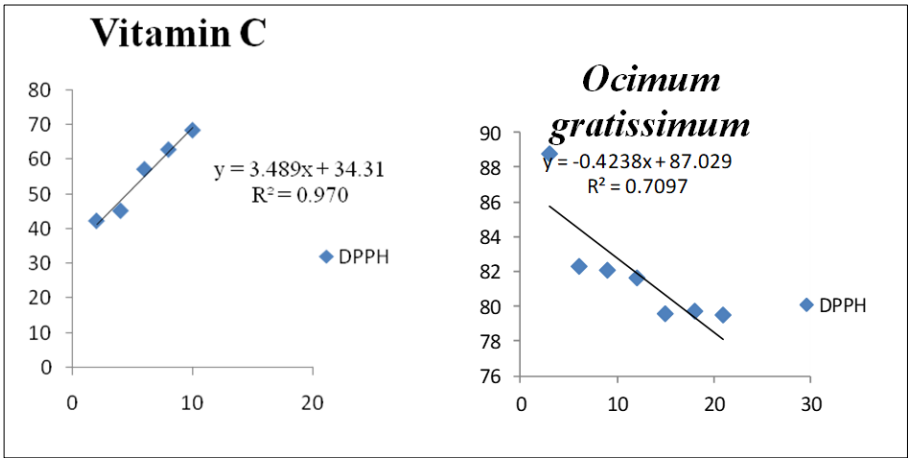

Fig 1: Calibration curve for DPPH activities for hot aqueous leaf extract of *Ocimum gratissimum* and ascorbic acid

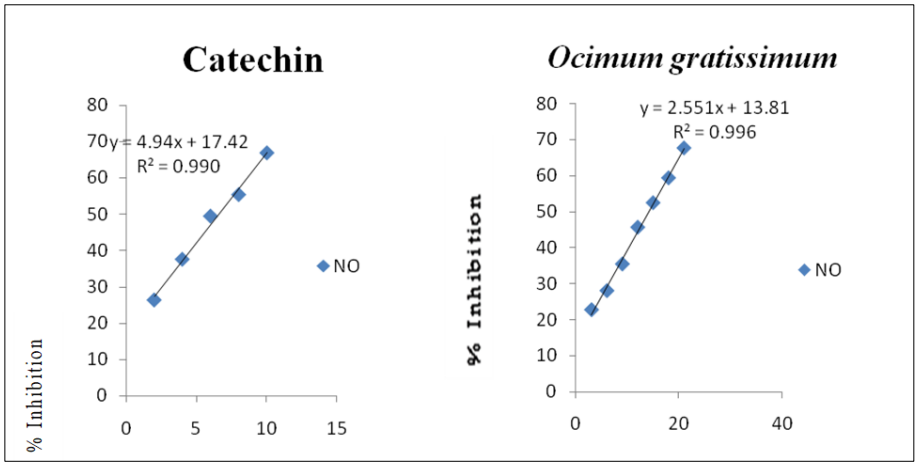

Fig 2: Calibration curve for nitric oxide activities for hot aqueous leaf extract of *Ocimum gratissimum* and catechin

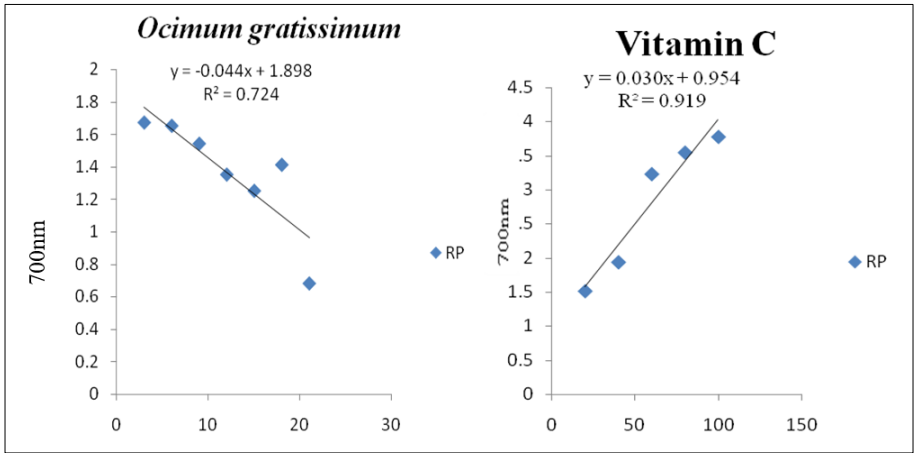

Fig 3: Calibration curve for reducing power activities for hot aqueous leaf extract of *Ocimum gratissimum* and ascorbic acid

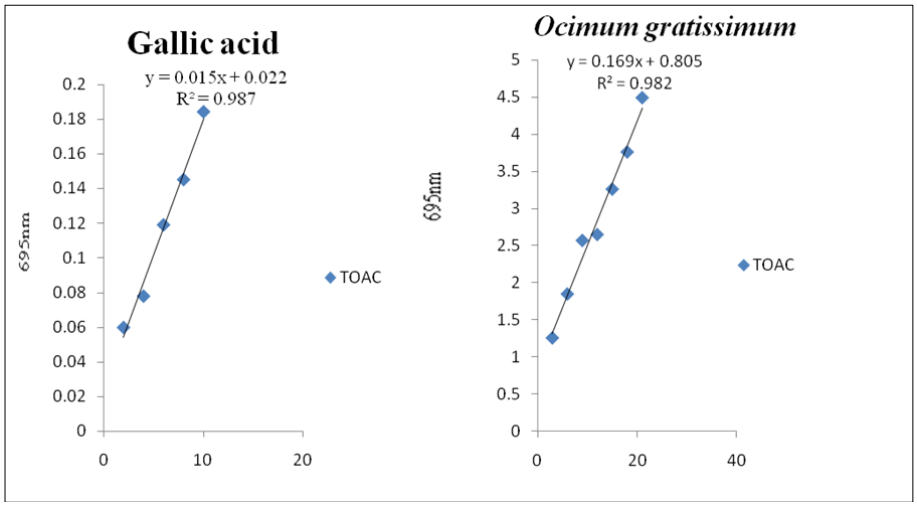

Fig 4: Calibration curve for total antioxidant capacity for hot aqueous leaf extract of *Ocimum gratissimum* and gallic acid.

4. Discussion

Therapeutic potentials of plants are due to their complex mixtures of different biological components. The effectiveness of medicinal plants is attributed to the action of these highly active phytochemicals (Asiwe *et al*., 2023, Enenebeaku *et al*., 2021) [8]. These plant chemicals are actually produced as secondary metabolites of plants as a means of protecting plants from diseases. They compounds that have considerable therapeutic potentials for human pathogens such as bacteria, fungi and viruses.

Phenolic compounds and some of their derivatives have been reported to be good antioxidants with high scavenging potentials against free radicals. Antioxidant properties of medicinal plants have been connected to the phytochemical compounds present in them (Aluko, 2021) [2] The occurrence of phytocompounds such as alkaloids, flavonoids, phenols, and tannins in hot aqueous leaf extract of *Ocimum gratissimum* may be a major contributing factor for the local usage of this particular plant in the treatment of various ailments. Reports have shown that phenols and flavonoids have a modulative effect on lipid peroxidation, which is involved in atherogenesis and carcinogenesis. (Asiwe *et al*., 2024) [5]. As polyphenolic compounds, tannins are possessing anti-diuretic and anti-diarrhoea potentials (Keyata *et al*., 2020) [15]. The phytochemical compounds present in this extract can synergistically contribute to the antioxidant properties of this plant. Alkaloids are responsible for the antimalarial, and antiasthma properties of plants as reported by Enenebeaku *et al*., 2021 [8].

Free radicals are by-products of several metabolic processes that occur in biological systems. They can cause enormous damage to tissues and cell biomolecules, leading to severe diseases such as diabetes, chronic inflammation, neurodegenerative disorders, and cancer (Ogidi *et al*., 2019) [20]. Although synthetic drugs are widely used to protect against oxidative damage, their use has been restricted due to side effects. Natural antioxidants from foodstuffs and therapeutic plants are an alternative to this problem.

The hot water leaf extract of the *Ocimum gratissimum* inhibits DPPH and shows its antioxidant activity. The DPPH test provides information on the reaction of a stable free radical DPPH compound with a strong 517nm absorption band in the visible region. When random electrons and free radical scattering are combined, the absorption rate drops and the DPPH solution decolorizes when the color changes from deep violet to light yellow. The reduction in absorption rate indicates the antioxidant potential of extracts.

The reducing power of a compound is related to the ability to transfer electrons, which could therefore be an important indicator of its potential antioxidant activity (Okokon *et al*., 2017) [21]. In this study, extracts compete with oxygen and interact with nitrogen oxides, inhibiting the generation of these atoms. The reducing ability of the extract of the extract occurred in a dose-dependent manner. The reducing ability of this extract can be attributed to the electron donor capacity of the compounds present in the extract. The reducing power activities exhibited by the extract might be due to the hydrogen donating ability of the leaf extracts. The results of

this study showed that reducing power decreased with increasing extract concentration. The result also showed that the total antioxidant capacity of *Ocimum gratissimum* hot water extract was significantly increased with increased extract concentrations. The decrease observed in the result trend may be due to the fact that the extract has exceeded its inhibitory capacity of 6% to 21%, which may indicate cytotoxicity.

Nitric oxide are unstable free radicals involved in many biological processes related to many diseases. It reacts with oxygen and produces stable nitrates through intermediates, high nitrogen oxide concentrations are toxic, and suppression of overproduction is an important goal (Wang *et al*., 2015). This study has shown that hot water extracts of leaves of *Ocimum gratissimum* may inhibit nitrogen oxide scavenging activity. The ability of extracts to clean nitrogen oxide emphasizes the importance of extracts in preventing the damages caused by NO to cells Enenebeaku *et al*., 2022 [9-10].

5. Conclusion

The results of this study show that the hot waterleaf extract of *Ocimum gratissimum* is rich in phytochemical components. Plant chemicals are responsible for the antioxidant properties of extracts. This shows that this plant is widely used for the treatment of various diseases. It is therefore recommended to clarify the mechanisms of action of these phytochemical compounds.

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