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Research Article

Evaluation of Nitrite Radical Scavenging Properties of Selected Zimbabwean Plant Extracts and Their Phytoconstituents

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Oxidative stress resulting from accumulation of reactive oxygen species has been associated with disease. The search for natural antioxidants of plant origin is necessitated by the side effects associated with synthetic antioxidants currently available. The objective of the study was to determine the antioxidant activity of *Combretum zeyheri*, *Combretum platypetalum*, and *Parinari curatellifolia* extracts by determining nitrite radical scavenging ability. The nitrite radical scavenging assay was used to evaluate the antioxidant potential of the extracts. The total flavonoid content of *P. curatellifolia* methanol extract was determined by the aluminium calorimetric method. The aqueous and ethanolic leaf extracts of *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia* extracts exhibited nitrite radical scavenging activity. The results show the scavenging activity in the order of potency: *P. curatellifolia* > *C. platypetalum* > *C. zeyheri* with concentration values of 103 μg/mL, 158 μg/mL, and 188 μg/mL for the ethanol extracts and 92.5 μg/mL, 97.5 μg/mL, and 198 μg/mL for the water extracts, respectively. *P. curatellifolia* ethanol extract was the most potent and the total flavonoid content was estimated to be 0.4 ± 0.05 mg/g quercetin and could account for the activity. Thus, our findings provide evidence that *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia* leaf extracts could be potential sources of natural antioxidants.

1. Introduction

Oxidation in living organisms is essential for the generation of energy during catabolism but these metabolic processes result in the continuous production of free radicals and reactive oxygen species (ROS) *in vivo*. Free radicals or more generally ROS are highly reactive species that are generated by cells during respiration and cell-mediated immune functions [1]. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, and pesticides [2]. The instability and reactivity of free radicals due to the lone electron in the outer shell can cause them to attack specific biomolecules in the body such as protein and lipids [3]. Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant mechanisms which scavenge/quench these free radicals preventing them from causing deleterious effects in the body [2]. The antioxidant mechanisms include endogenous and exogenous systems such as catalase and vitamin antioxidants, respectively. When the generation of free radicals exceeds the scavenging capacity of the cell's endogenous systems, the excess free radicals seek stability through electron pairing with biological macromolecules of healthy cells such as proteins, lipids, and DNA. The pairing of the free radicals with biomolecules can eventually result in the induction of lipid peroxidation which leads to cancer, atherosclerosis, cardiovascular diseases, ageing, and inflammatory diseases [2, 4]. Prolonged oxidative stress can result in permanent damage to vital body organs, which could eventually lead to chronic disorders such as heart diseases, diabetes, cirrhosis, malaria, neurodegenerative diseases, AIDS, cancer, and premature aging [3, 5]. It has been

noted that about 95% of the pathologies observed in people above 35 years of age are associated with production and accumulation of free radicals [6].

Nitric oxide (NO) and reactive nitrogen species (RNS) are free radicals that are derived from the interaction of NO with oxygen or reactive oxygen species [7]. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals such as superoxide [8]. NO is synthesized by three isoforms of the enzyme nitric oxide synthase (NOS), endothelial NOS, neuronal NOS, and inducible NOS (iNOS). Nitric oxide (NO) is generated from amino acid L-arginine by the enzymes in the vascular endothelial cells, certain neuronal cells, and phagocytes [3]. Low concentrations of NO are sufficient in most cases to effect the physiological functions of the radical. NO is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation, and antimicrobial and antitumor activities [9]. Chronic exposure to nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis. The toxicity of NO increases greatly when it reacts with the superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) [8]. Nitric oxide has been shown to be directly scavenged by flavonoids [10].

Natural antioxidants are considered to be safe and bioactive [11]. The antioxidants from natural sources are the only alternative to synthetic antioxidants in counteracting the free radicals associated disease [3]. The antioxidant activities of phenolic compounds are mainly due to the redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers, in addition to their metal chelating potential. The antioxidant activity of phenolics plays an important role in the adsorption or neutralization of free radicals [9]. *In vitro* quenching of NO radical is one of the methods that can be used to determine antioxidant activity [3]. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions [12].

In recent years, various species of plants have been used in preparation of drugs and are consumed as food due to their antioxidant activities [13]. The extracts of medicinal plants and natural products have become a great source of antioxidant and antiageing properties [14]. Recently, much attention has been directed towards the development of ethnomedicines with strong antioxidant properties but with low cytotoxicity [15]. Therefore, antioxidants with free radical scavenging activities of medicinal plants may have great relevance in the prevention of diseases and in therapeutic properties [16]. Plants, rich in their phytochemical compounds, are good sources of antioxidants and radical scavengers [5]. *Combretum platypetalum* has been used traditionally for swelling caused by mumps, pneumonia, abdominal pains, diarrhoea, antiemetic, dysmenorrhoea, infertility in women, earache, epistaxis, and haemoptysis [17]. *Parinari* species, such as *P. curatellifolia* and *P. excelsa*, are traditionally used in Africa as a remedy for dysentery, epilepsy, malaria, toothache, and venereal diseases [18]. The leaves of *P. curatellifolia* are indicated to treat stomach aches in Southern Uganda [19]. *Combretum zeyheri* has been used traditionally to treat conditions such as toothache, cough, scorpion bite, bloody diarrhoea, arrest of menstrual flow, and abdominal pain.

Modern pharmaceuticals and nutriceuticals are currently out of reach of a large proportion of the human population in developing countries [20]. This necessitates the use of other sources of human knowledge to provide common health benefits. Thus, herbal medicines are now regarded as important but underutilized tool against the disease [21]. The widespread use of *Combretum* species and *Parinari* species in indigenous medicine for many different ailments exhibited by several species and the need for effective antioxidant agents justify the further investigation of the antioxidant activity of *Combretum* species and *Parinari* species that are found in Zimbabwe. The main objective of the study was to determine the antioxidant activity of *C. zeyheri*, *C. platypetalum,* and *P. curatellifolia* leaf extracts and *P. curatellifolia* phytoconstituents by nitrite radical scavenging assay.

2. Materials and Methods

2.1. Materials. All chemicals used were purchased from Sigma-Aldrich chemical company (Darmstadt, Germany). These were ethanol, methanol, N-butanol, ammonia, aluminium chloride, potassium acetate, quercetin, gallic acid (GA), sulphanilamide, naphthalene diamine dihydrochloride, phosphoric acid, sodium nitroprusside (SNP), KH_2PO_4 (monobasic anhydrous), Na_2HPO_4 (dibasic anhydrous), sodium chloride (NaCl), and potassium chloride (KCl).

2.2. Collection and Preparation of Plant Material. Leaves of *Combretum zeyheri*, *Combretum platypetalum*, and *Parinari curatellifolia* were collected from Norton, Mashonaland Central, Zimbabwe. The plants were classified by Mr. Christopher Chapano, a taxonomist at the National Herbarium and Botanic Gardens (Harare, Zimbabwe). The plant leaves were dried in a Labcon orbital incubator (Labotech Co., Cape Town, South Africa). The plants were stored in the Biomolecular Laboratory at the Department of Biochemistry, University of Zimbabwe.

2.3. Preparation of Extracts. For the ethanol extracts, dried leaves of the plant species were ground in a two-speed blender (Cole Palmer Instrument Co., USA) and the samples were extracted with ethanol on an orbital shaker (Bibby Scientific Limited, Staffordshire, UK) using a 4 : 1 solvent to dry weight ratio for 10–15 mins. The extract was filtered through a Whatman filter paper and the filtrate was decanted into preweighed labelled containers. The filtrate was concentrated under a fan and then weighed and percentage yield was calculated. For the water extracts approximately 1 g of dry fine powder was suspended in 10 mL distilled water. The extract solution was stirred magnetically for 24 hrs at room temperature on a magnetic stirrer (Minor-2 Voss Instruments

Ltd., Malden, Essex). The extracts were then filtered using a Whatman number 1 filter paper. The filtrate was concentrated to dryness in a Labcon Incubator (Labotec Co., Cape Town, South Africa) to obtain the crude extracts.

2.4. Isolation of Phytoconstituents. The phytoconstituents were isolated from *Parinari curatellifolia* [22]. Alkaloids were isolated by mixing 1 g of powdered sample with 1 mL of 10% (v/v) ammonia solution and extracted with 5 mL ethanol for 10 mins on a water bath at 40°C. The extracted solution was then filtered through Whatman filter paper No. 1 and the filtrate was concentrated using air drying under a fan. Isolation of flavonoids was achieved by heating 1 g of powdered sample with 5 mL methanol on a water bath at 40°C for 10 mins. The filtrate was then concentrated to 1/4th of its original volume and was dried under a fan to a powdered extract. For saponins, 1 g of powdered sample was extracted with 5 mL methanol by heating on a water bath at 40°C for 10 minutes. The extract was filtered and evaporated to 1 mL, mixed with 0.5 mL water, and then extracted thrice with 3 mL n-butanol. The solution was allowed to separate into phases. The aqueous phase was decanted and the n-butanol phase was evaporated and concentrated under a fan. Aluminium chloride colorimetric method was used for flavonoid determination [23]. Each plant extract (0.5 mL) of 1, 2, 4 mg/mL methanol was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. It remained at room temperature for 30 mins. A volume of 150 μL was pipetted in a 96-well plate and the absorbance of the reaction mixture was measured at 415 nm in a SpectraMax Plus microplate reader (Molecular Devices, CA, USA). The calibration curve was prepared by preparing quercetin solutions at concentrations of 6 to 100 mg/mL in methanol. The quantity of flavonoids was interpolated from the calibration curve of quercetin using GraphPad Instat software (GraphPad Prism Inc., San Diego, CA, USA).

2.5. Nitric Oxide Radical Scavenging Assay. The assay is the nitric oxide radical scavenging assay [24]. The extracts were prepared from a 10 mg/mL ethanol crude extract. These were then serially diluted with distilled water to make concentrations from 100–1000 μg/mL of the three plants and the standard gallic acid. These were stored at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the ethanol extracts (100–1000 μg/mL) and incubated at 25°C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The colour tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150 μL of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at 546 nm using a SpectraMax Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the ethanol extracts and gallic acid were calculated using the following formula:

percentage nitrite radical scavenging activity:

$$\text{nitric oxide scavenged } (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100, \quad (1)$$

where A_{control} = absorbance of control sample and A_{test} = absorbance in the presence of the samples of extracts or standards.

2.6. Statistical Analysis. Data analyses were performed using GraphPad Instat software (GraphPad Prism Inc., San Diego, CA, USA). Levels of significance were determined using ANOVA using the Dunnet post test where all columns of treatments were compared to the control. All data were expressed as mean ± standard deviation. $P \leq 0.05$ values or less were considered to indicate statistically significant difference.

3. Results

3.1. Quantification of Flavonoids Isolated from P. curatellifolia Dried Leaves. The total flavonoid content for methanolic extracts of *P. curatellifolia* was found to be 0.4 ± 0.05142 mg/g of quercetin equivalent.

3.2. Nitrite Radical Scavenging Assay

3.2.1. Ethanol Extracts of C. zeyheri, C. platypetalum, and P. curatellifolia. Nitrite radical scavenging assay was carried out on the ethanol extracts of *C. zeyheri*, *C. platypetalum,* and *P. curatellifolia* dried leaves from a concentration of 100 to 1000 μg/mL. Percentage free radical scavenging was plotted against concentration of the extracts as shown in Figure 1. The plants exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite radical which was generated from SNP at physiological pH in an aqueous environment. The antioxidant activity increased with an increase in concentration of the extracts reaching a plateau. Increasing the concentration of the extracts did not result in an increase in the nitrite radical scavenging activity. The maximum free radical scavenging activity and potency were interpolated from Figure 1 to give results as shown in Table 1. *P. curatellifolia* ethanol extract was the most potent as it removed the nitrite radical at a lower concentration as compared to the other plant extracts. *P. curatellifolia* had a maximal scavenging activity at 103 μg/mL followed by *C. platypetalum* which was at 158 μg/mL and *C. zeyheri* at 188 μg/mL.

3.2.2. Water Extracts of C. zeyheri, C. platypetalum, and P. curatellifolia. The nitrite radical scavenging assay was carried out on the water extracts from a concentration range of 100 to

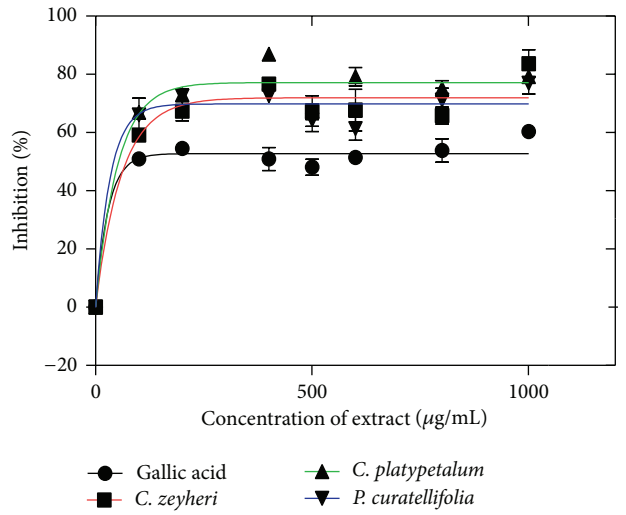


Figure 1: Nitric oxide scavenging activity of ethanol extracts from dried leaves of *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia*.

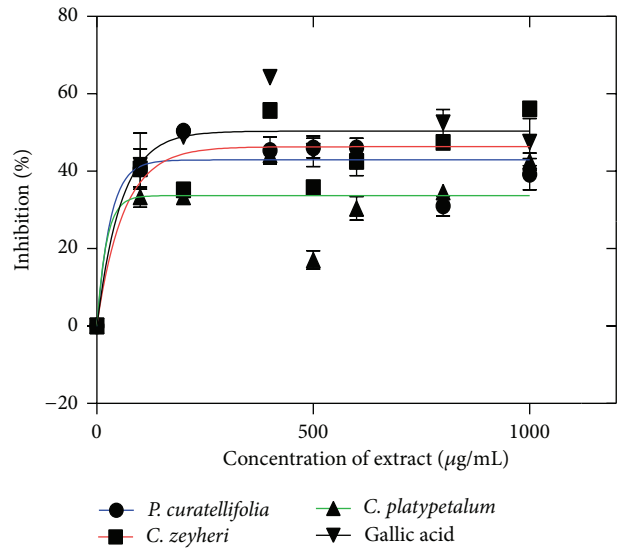


Figure 2: Nitric oxide scavenging activity of water extracts from leaves of *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia*.

1000 μg/mL. The percentage radical scavenging of the nitrite radical by the water extracts is shown in Figure 2. The water extracts exhibited less free radical scavenging capacity than the ethanol extracts. The trend of antioxidant activity was the same for both ethanol and water extracts with *C. platypetalum* showing the highest capability of quenching the NO radical followed by *C. zeyheri* and *P. curatellifolia*, respectively, as the results in Table 1 indicate. The ethanol extracts results were used to determine potency and maximum percentage scavenging of the plants since they exhibited the greatest scavenging activity as compared to the water extracts. The potency of the extracts was interpolated from the graph in Figure 1. The summary of the results is shown below in Table 1.

Table 1: Nitrite radical scavenging activity of gallic acid and *Combretum zeyheri, Combretum platypetalum,* and *Parinari curatellifolia* extracts.

Plant	Ethanol extracts μg/mL	Water extracts μg/mL	Nitric oxide scavenged %
C. zeyheri	188	198	72.5
C. platypetalum	158	97.5	77.5
P. curatellifolia	103	92.5	70
Gallic acid	87.5	173	53.5

3.2.3. Isolated Phytoconstituents from P. curatellifolia. The nitrite radical assay was carried out on the isolated phytochemicals from the most potent plant *P. curatellifolia*. The assay was done on saponins, alkaloids, and flavonoids from a concentration range of 100 to 1000 μg/mL. The results were analysed using GraphPad Prism 5 software and these are shown in Figure 3 and the values were used to determine potency and the greatest percentage radical scavenging of the phytochemicals. The results are shown in Table 2. The results showed that the saponins were the most potent followed by flavonoids and lastly alkaloids. The maximal scavenging activity was shown by alkaloids, then saponins, and lastly flavonoids.

4. Discussion

It is widely recognized that many of today's diseases are due to the oxidative stress that results from an imbalance between formation of ROS/RNS and their neutralization when endogenous antioxidant mechanisms are unable to quench the free radicals [25]. The free radicals are known to be scavenged by synthetic antioxidants, but due to their adverse side effects leading to carcinogenicity, search for effective and natural antioxidants has become crucial [26]. Natural antioxidants are believed to be safer and bioactive [12].

Nitric oxide (NO) is generated from amino acid L-arginine by vascular endothelial cells, phagocytes, and certain cells of the brain. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals. The toxicity of NO becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion ($ONOO^-$) [3].

The antioxidants from natural sources could be the alternative to synthetic antioxidants in counteracting oxidative stress associated diseases. A great number of naturally occurring substances have been recognized to have antioxidant abilities and various *in vitro* methods have been used to assess their free radical scavenging and antioxidant activity. Therefore, in the present study, *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia* at different concentrations were assessed for their nitrite free radical scavenging activity in an *in vitro* model. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize with sulphanilamide acid and couple with naphthyl ethylenediamine, forming pink colour, which was measured at 546 nm

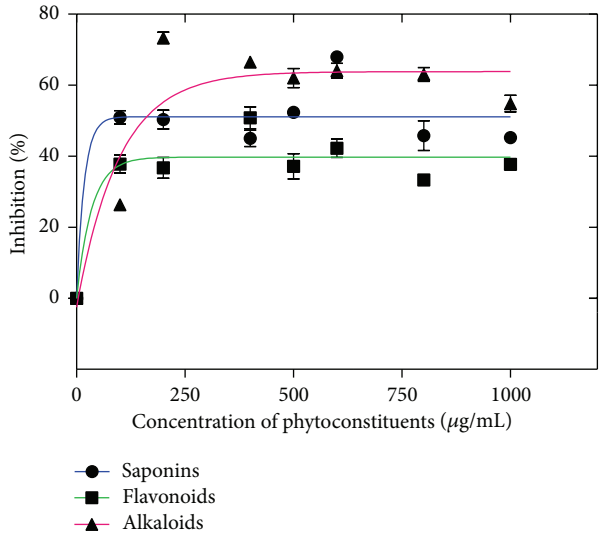


Figure 3: Nitric oxide scavenging activity of the isolated phytoconstituents extracts from leaves of *P. curatellifolia*.

Table 2: Nitrite radical activity of *Parinari curatellifolia* phytoconstituents.

Phytoconstituents	Potency (μg/mL)	Nitric oxide scavenged %
Alkaloids	197	64
Saponins	62	52
Flavonoids	78	41

[24]. As antioxidants donate protons to the nitrite radical, the absorbance is decreased. The decrease in absorbance was used to measure the extent of nitrite radical scavenging [27].

It was observed that all the extracts both ethanol and water scavenged nitrite radical in a dose dependent manner and reached a plateau in which a further increase in the concentration of the extracts did not result in an increase in the quenching of the nitrite radical. Hence, *C. zeyheri, C. platypetalum*, and *P. curatellifolia* leaf extracts have antioxidant activity, particularly when compared to the positive control, gallic acid. Figures 1 and 2 illustrate that there is a significant decrease in the NO radical due to the scavenging ability of extracts and gallic acid. Based on the results, the ethanol extracts of the three plants were found to be the more effective nitric oxide scavengers than the water extracts. The activity of the ethanol extracts could be attributed to the components such as phenolic compounds that are isolated by each solvent. Polyphenols are considered one of the major antioxidant components in plant extracts [28]. Ethanol extracts are thought to contain more polyphenols compared to aqueous extracts as ethanol is more efficient at penetrating the cellular membrane to extract the intracellular ingredients from the plant material resulting in more efficient extraction of polyphenols [29]. The decrease in activity of the aqueous extract could be ascribed to polyphenol oxidase, which degrades polyphenols in water extracts but is inactive in methanol and ethanol [29]. Previous studies by Masoko and Eloff have shown that ethanol extracted the highest quantity of antioxidant compounds based on the DPPH-TLC analysis they conducted [2].

When comparing the three extracts with standard gallic acid the ethanol extract of *P. curatellifolia* was the most potent nitric radical scavenger than that of the other two extracts *C. zeyheri* and *C. platypetalum*. The values of potency as shown in Table 1 are as follows: *P. curatellifolia* > *C. platypetalum* > *C. zeyheri*. The values are 103 μg/mL, 158 μg/mL, and 188 μg/mL, respectively. The same trend was observed with the water extracts and the potency results are 92.5 μg/mL, 97.5 μg/mL, and 198 μg/mL, respectively. The ethanol extracts inhibited the nitrite radical more than the standard gallic acid and the water extracts inhibited the nitrite radical less than the gallic acid. Although the ethanol extracts scavenged the nitrite radical at higher concentrations, gallic acid was more potent as shown by the value which was 87.5 μg/mL; this showed that it inhibited the nitrite radical at lower concentrations.

Chronic exposure to nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis [30]. In view of that the study has shown that these three plant extracts, both ethanol and water, can be used as sources of antioxidants. A study by Masoko and Eloff showed that the leaves of the Combretaceae family are known for their pharmacological activity and that various solvents extracted antioxidant compounds from the leaves of the plants belonging to members of the Combretaceae family [2].

After discovering that *P. curatellifolia* was more potent, phytoconstituents were isolated from it. The phytoconstituents saponins, alkaloids, and flavonoids were isolated from *P. curatellifolia* by different methods and their nitrite radical scavenging activity was determined. Saponins were the most potent followed by flavonoids and then alkaloids at concentration values of 61.6 μg/mL, 77.5 μg/mL, and 197 μg/mL, respectively.

Flavonoids are one of the most diverse and widespread groups of natural compounds and are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [29]. The assumption that other phytochemicals had been extracted was made; hence there was a need to quantify the flavonoids that were present in the crude extract of *P. curatellifolia*. The total flavonoid content of the methanolic extract of *P. curatellifolia* dried leaves was 0.4 ± 0.05142 mg/mL quercetin equivalent. A study by Ogunbolude et al. showed that the flavonoid content of *P. curatellifolia* seeds was 1.57 ± 00.17 mg/g quercetin equivalent [31]. The difference could be as a result of the differences in the plant part that was used, seeds as compared to the leaves. The difference in geographical location in the same species of a plant could also explain the differences.

P. curatellifolia is thought to utilize various antioxidative mechanisms which include free radical scavenging, reduction, and deactivation by chelation of transition metals involved in the initiation of free radical induced macromolecular damage. Our results indicate that *P. curatellifolia* is a potent scavenger of the nitrite radical. The phytoconstituents isolated from *P. curatellifolia* have all been shown in several

studies to have antioxidant activity as well. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [32]. Preliminary phytochemical screening on *P. curatellifolia* indicated the presence of antioxidant compounds such as polyphenols [33]. The ethanolic extracts of the seeds contained polyphenols, flavonoids, vitamin C, and nonprotein thiols such as glutathione. Glutathione and vitamin C are well-known antioxidants that are found in plants and provide exogenous antioxidant mechanisms to cells. NO has been found to be directly scavenged by flavonoids [11]. The presence of flavonoids and saponins could explain why *P. curatellifolia* was more potent at quenching the NO radical than the other plants *C. zeyheri* and *C. platypetalum* in this study.

5. Conclusion

In conclusion, the results obtained in the present study indicated that ethanol and water extracts of *C. zeyheri*, *C. platypetalum*, and *P. curatellifolia* scavenged the nitrite radical showing that they are potent antioxidants. Hence, these plant extracts can be used as natural sources of antioxidants as they could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. They also have potential application in industry as natural antioxidants that could be used as food additives to prevent food deterioration as synthetic antioxidants that are available are associated with a lot of side effects.

Abbreviations

BHA:	Butylated hydroxyl anisole
BHT:	Butylated hydroxyl toluene
DPPH-TLC:	2, 2-Diphenyl-1-picryl hydrazyl-thin layer chromatograph
GA:	Gallic acid
GPx:	Glutathione peroxidase
GSH:	Glutathione
iNOS:	Nitric oxide synthase (iNOS)
NADH:	Nicotinamide adenine dinucleotide hydrogen
NO:	Nitric oxide
PG:	Propyl gallate
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species
SOD:	Superoxide dismutase
SNP:	Sodium nitroprusside.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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