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Phytochemical Screening and *In vitro* Antioxidant Assessment of *Cassia alata* (Linn) Leaf Extracts

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ABSTRACT
This study investigated the phytochemical components and assessed the antioxidant activity of *Cassia alata* leaf extracts. The results showed that alkaloids were present in all the extracts, while saponins, phenols and flavonoids were detected in ethyl acetate and methanol extracts. The antioxidant capacities of the extracts, determined using four models in the presence of vitamin C and quercetin as standards at four different concentrations (0.25, 5.0, 1.0 and 2.0 mg/L), demonstrated that the activities were concentration-dependent in all the extracts. Thus, *Cassia alata* having important phytochemicals may be used in therapeutic medicine as a source of natural antioxidants.

Keywords: Antioxidant activity, *Cassia alata*, Maceration, Phytochemical screening

INTRODUCTION
Medicinal plants have played an important role as the source of anticancer agents (Kumar *et al*., 2011). Perhaps this has become possible due to the presence of a wide variety of phenolic compounds such as flavonoids in those plants and their attendant traditional use as antioxidants to scavenge free radicals and inhibit lipid peroxidation (Kumawat *et al*., 2012). There has been an increasing interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical-induced tissue injury. As a result, many vegetables, fruits and other plant species are already commercially exploited either as antioxidants or are being investigated for novel antioxidants (Koleva *et al*., 2002).

Cassia alata (Linn), locally known as 'asunrun oyinbo' in yoruba and 'ogalu' in Igbo, belongs to the Fabaceae family. It is an ornamental shrub or tree that grows up to 12 m high and is widely found in most tropical countries with warm and humid environments such as Southeast Asia and Africa (Halim-Lim *et al*., 2020). This tree, besides its uses as a source of firewood and timber, has applications in folkloric medicine (El-Mahmood and Doughari, 2008). In Ghana and Ivory Coast, decoctions of the leaves are used to treat diarrhea, dysentery and other gastrointestinal problems. The macerated juices of the young fresh leaves are used to treat urinary infections and asthma (El-Mahmood and Doughari, 2008). Previous reports in scientific literature indicated that the leaves of *C. alata* (Linn) can be used as a remedy for boils, wound infections, diarrhea, gastrointestinal tract infections and scarlet fever (Benjamin and Lamikanra, 1981). Recent reports have documented additional pharmacological activities of *C. alata* leaf which includes antiviral (Shaheen *et al*., 2015) and DPPH radical scavenging activities (Chatterjee *et al*., 2013). This study focuses on screening of the phytochemicals present in three extracts of *C. alata* leaf and further evaluates the antioxidant activities of the extracts.

MATERIALS AND METHODS
Plant Material
Cassia alata leaves were collected from Finima, Bonny local government area, Rivers State, Nigeria. The sample was identified by Prof. (Mrs.) O. B. Green of the Department of Plant Science and Biotechnology, Rivers State University, Nigeria. The plant material was sorted out to obtain only fresh leaves and washed with distilled water (without squeezing) to remove debris and dust particles. It was dried under the shade to prevent ultra-violet rays from inactivating the chemical components (Das *et al*., 2010; Ncube *et al*., 2008). The dried leaves were later pulverized using a manual blender.

Extraction
Maceration method was adopted for this work. Hence, the powdered leaves were extracted using *n*-hexane, ethyl acetate and methanol in increasing order of polarity. The powdered leaves (600 g) were weighed accurately and soaked in 2 L of *n*-hexane in a stoppered flask at room temperature for 24 hours with frequent agitation (Ndukwe *et al*., 2015; Handa *et al*., 2008). After 24 hours, the mixture was filtered to obtain the *n*-

hexane extract. The residue left was again subjected to second successive extraction with another batch of *n*-hexane according to the procedure described above to obtain the second batch of n-hexane extract. This process was performed four times. The same procedure was performed on the plant residue using 1.5 L of ethyl acetate and 1.27 L of methanol in sequence. The three extracts obtained were then separately concentrated using a rotary evaporator (Labrota 4002) at 40 °C and air-dried.

Phytochemical Screening

Phytochemical screening of the extracts was carried out according to standard procedures reported in scientific literature (Ndukwe *et al*., 2016; Khan *et al*., 2011) to detect the presence of alkaloids, flavonoids, phenols, saponins, tannins, terpenoids and cardiac glycosides.

Antioxidant Assays

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity was determined according to the method reported by Sunil and Ignacimuthu (2011) with few modifications. 1 ml of methanolic DPPH (0.15%) was mixed with 3 ml of each extract at varying concentrations (0.25, 5.0, 1.0 and 2.0 mg/L) or reference antioxidant (vitamin C or quercetin) and incubated in a dark room for 30 minutes. Thereafter, absorbance was measured at 515 nm using a UV-Vis spectrophotometer (UV-2500). The DPPH radical scavenging/inhibition activity was expressed as a percentage and calculated using equation 1. Distilled water was used as blank.

$$\% \text{ Scavenging Activity} = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

Where $\mathbf{A_c}$ is absorbance of control, $\mathbf{A_s}$ is absorbance of sample.

Reducing Ability

The ability of the extracts to reduce iron (III) ion was investigated according to Oyaizu (1981) method as modified by Okoko and Diepreye (2012). Thus, 0.5 ml of each extract at varying concentrations (0.25, 5.0, 1.0 and 2.0 mg/L) or reference antioxidant (vitamin C or quercetin) was mixed with 0.5 ml of phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of potassium ferricyanide (1%) and incubated at 50 °C. After incubation for 20 minutes, 0.5 ml of trichloroacetic acid (10%) was added and centrifuged for 10 minutes at 3000 rpm. A portion of the upper layer (0.5 ml) was mixed with 0.5 ml distilled water and 0.1 ml ferric chloride (0.1%). After 10 minutes of incubation at room temperature, absorbance was measured at 700 nm using a UV-Vis spectrophotometer (UV-2500). An increase in absorbance showed greater reducing ability.

Hydrogen Peroxide Inhibitory Assay

Hydrogen peroxide scavenging activity was carried out by replacement titration, as reported by Zhao *et al*. (2006) with some modifications. Briefly, 1 ml of hydrogen peroxide (0.1 M), 1 ml of each extract at varying concentrations (0.25, 5.0, 1.0 and 2.0 mg/L) or reference antioxidant (vitamin C or quercetin), 3 ml of ammonium molybdate (3%), 10 ml of 2 M sulphuric acid and 7 ml of potassium iodide (1.8 M) were introduced into a conical flask. This was then titrated against sodium thiosulphate (5.09 M). The absorbance of the mixture was read at 760 nm using a UV-Vis spectrophotometer. The hydrogen peroxide scavenging activity was calculated in a similar way using equation 1 with distilled water as blank.

Hydroxy Radical Scavenging Assay

Hydroxy radical scavenging activity was investigated using the method described by Bera *et al*. (2015) with few modifications. 1 ml of phosphate buffer (0.2 M, pH 7.2), 1 ml of test solution either extract (0.25, 5.0, 1.0 and 2.0 mg/L) or reference antioxidant (vitamin C or quercetin), 0.02 ml of ferric chloride (0.02 M) and 0.05 ml of phenanthroline (0.04 M) were introduced into a test tube. The reaction was initiated by adding 0.05 ml of 7 mM hydrogen peroxide. After 5 minutes of incubation at room temperature (25 °C), absorbance was measured at 560 nm using a UV-Vis spectrophotometer (UV-2500). Hydroxy radical scavenging activity was calculated in a similar way using equation 1 with methanol as blank.

Statistical Analysis

Statistical comparisons of the percentage values were calculated using linear regression analysis. All linear regression in this study were analyzed using origin 6.0 professional software.

RESULTS AND DISCUSSION

Phytochemical Constituents

Qualitative screening of methanol and ethyl acetate extracts indicated the presence of alkaloids, saponins, flavonoids and phenols, whereas, tannins, terpenoids and cardiac glycosides were absent. The *n*-hexane extract indicated the presence of only alkaloids (Table 1). The phytochemical profile exhibited by the methanol extract of *C. alata* leaf agreed with the reports of El-Mahmood and Doughari (2008) and Idu *et al.* (2007), all of whom linked the antimicrobial activity of this plant to the presence of bioactive secondary metabolites such as alkaloids, saponins, flavonoids and phenols. These phytochemicals are known to possess a variety of biological activities including antimicrobial, antioxidant, anti-inflammatory and anticancer activities (Ndukwe *et al*., 2020).

Antioxidant Activity

Since there are many methods used to evaluate free radical scavenging activity of compounds (Paulova *et al*., 2004) and the antioxidant activity of plant extracts varies with the assays due to the complex nature of phytochemicals present in them as well as the solvent used for extraction (Ndukwe *et al*., 2020; Rakholiya *et al*., 2011); therefore, it is reliably important to use several analytical methods to corroborate the findings of each method. Hence, DPPH radical scavenging assay, hydroxy radical inhibitory assay, hydrogen peroxide scavenging assay and ferric reducing power models were used for the antioxidant assay in this study.

The DPPH radical scavenging assay evaluates the ability of extracts to donate hydrogen or to scavenge free radicals. The percentage of inhibition was measured to determine the antioxidant activity of the extracts. Four varying concentrations (0.25, 5.0, 1.0 and 2.0 mg/L) of the different extracts of *C. alata* leaves showed different percentages of inhibition (Figures 1-3). Interestingly, the scavenging activity of each extract increased in a concentration-dependent manner, however, at 2.0 mg/L, the extracts showed much lower DPPH scavenging activities (20% - 27%) than the standards (35% - 50.2%). The DPPH radical scavenging activity of *C. alata* extracts were subjected to regression line equation ($y = 4.313x + 15.957$ with $R^2 = 0.7959$, $y = 5.0087x + 11.304$ with $R^2 = 0.92$ and $y = 1.9826x + 23.391$ with $R^2 = 0.8072$; Figures 1-3). Scavenging activities of the three extracts were compared with those of the standards. It was clear from the Pearson's correlation coefficient that there was a significant positive linear relationship with the concentrations of the extracts used, indicating that there are scavenging activities in the extracts of the leaves of *C. alata*. This result is consistent with a previous report by Sagbo *et al*. (2017) which showed that the scavenging effects on DPPH radical increased with increasing concentration of extracts and standards. This activity can be attributed to their ability to provide hydrogen to DPPH free radicals in order to stabilize them (Kasangana *et al*., 2015).

Table 1: Phytochemicals present in *Cassia alata* leaf

Phytochemicals	*n*-Hexane extract	Ethyl acetate extract	Methanol extract
Alkaloids	+	+	+
Saponins	-	+	+
Tannins	-	-	-
Flavonoids	-	+	+
Phenols	-	+	+
Terpenoids	-	-	-
Cardiac glycosides	-	-	-

Key: + Present, - Absent

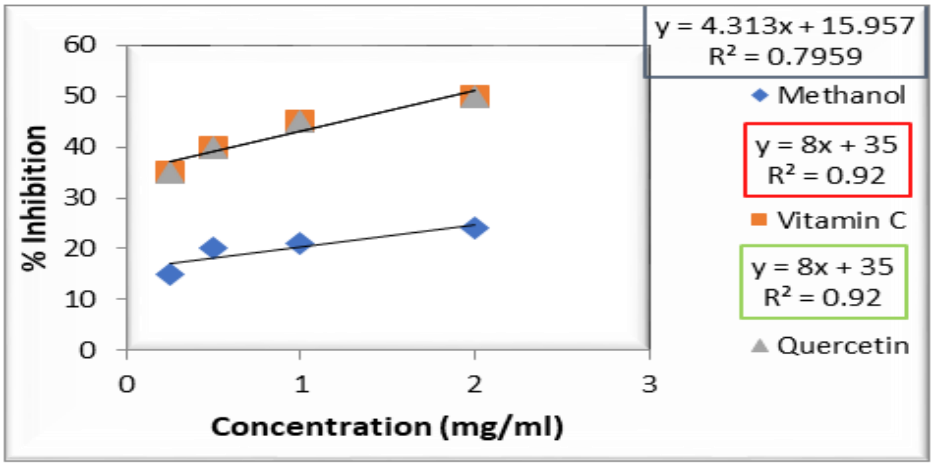

Figure 1: DPPH free radical scavenging activity of the methanol extract of *C. alata* leaf

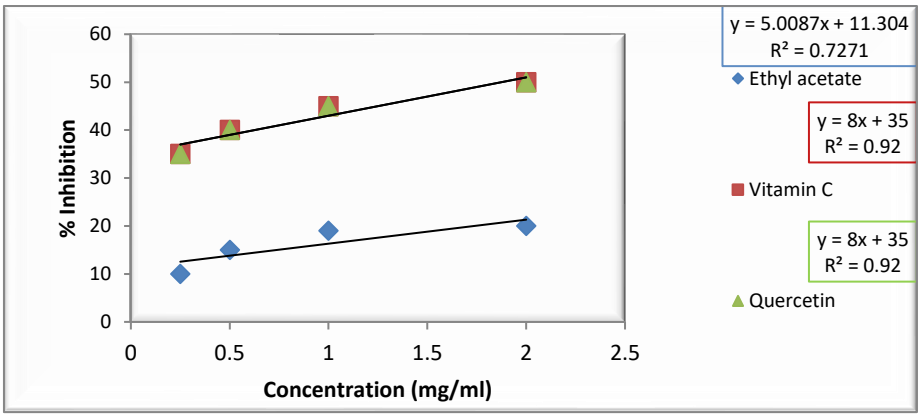

Figure 2: DPPH free radical scavenging activity of the ethyl acetate extract of *C. alata* leaf

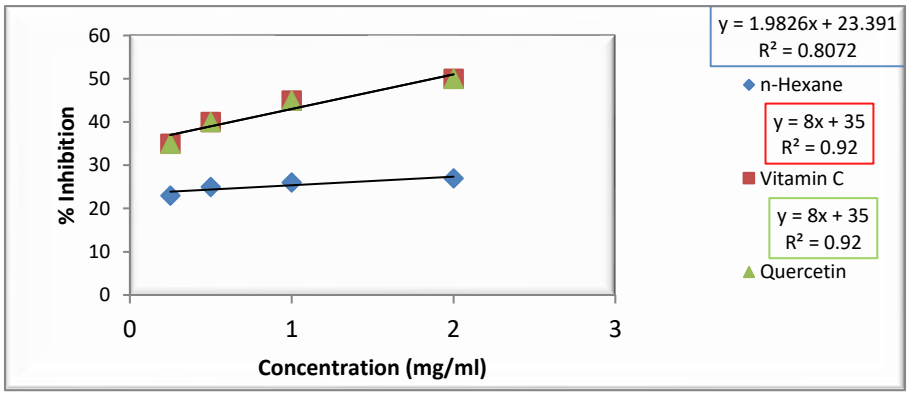

Figure 3: DPPH free radical scavenging activity of the *n*-hexane extract of *C. alata* leaf

The ferric reducing ability of the extracts was studied by measuring the transformation from Fe^{3+} to Fe^{2+} in the presence of *C. alata* leaf extracts. In this study, a concentration-dependent increase in the reducing ability for the plant extracts and standards (vitamin C and quercetin) was observed (Figures 4-6); indicating the potential of the extracts to reduce oxidative stress. The *n*-hexane and methanol extracts exhibited strong significant correlations since the results were almost on a par with quercetin (R^2 = 0.8995, R^2 = 0.9441 and R^2 = 0.92, Figures 6 and 4). In addition, the ethyl acetate extract was determined to have the same antioxidant efficiency with that of the standard vitamin C (R^2 = 0.6116 and R^2 = 0.6403 respectively, Figure 5). However, studies have also suggested that plant secondary metabolites such as phenol and flavanol are largely involved in the antioxidant activities of methanol and ethyl acetate extracts (Perez-Guiterrez *et al.*, 2016; Sudha and Srinivasan, 2014;). Therefore, this finding supports the result of Jack *et al.* (2020) who reported that the reducing power of plants correlates with its phenolic content.

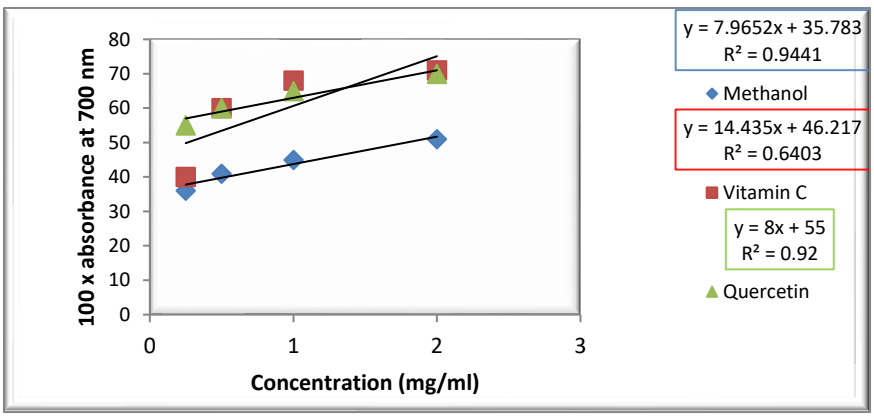

Figure 4: Reducing ability of the methanol extract of *C. alata* leaf

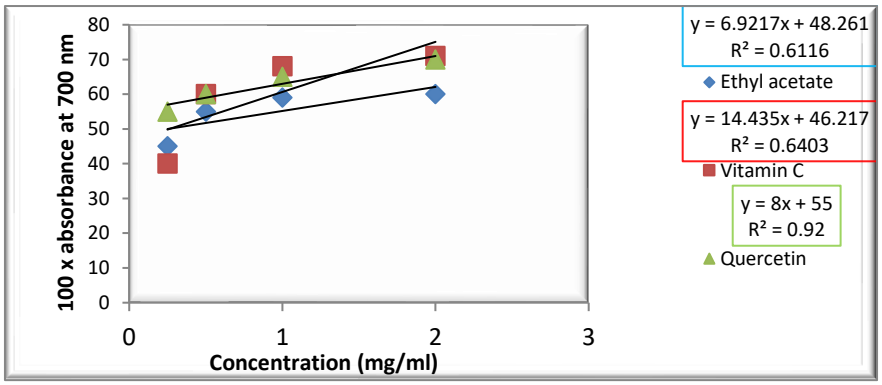

Figure 5: Reducing ability of the ethyl acetate extract of *C. alata* leaf

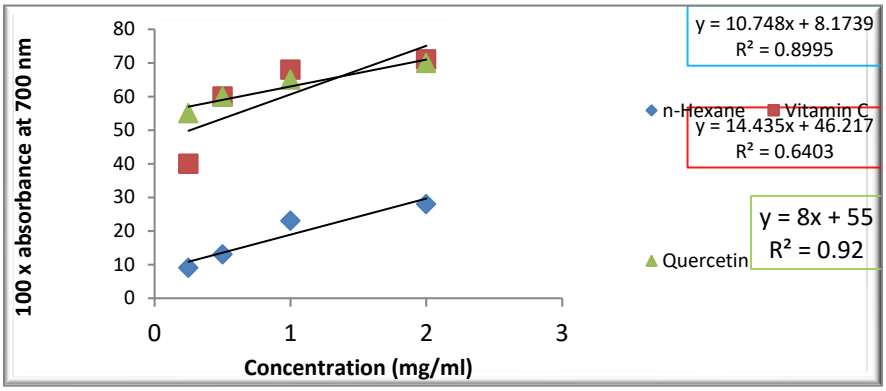

Figure 6: Reducing ability of the *n*-hexane extract of *C. alata* leaf

H_2O_2 is an important reactive oxygen species based on its ability to cause oxidative degradation of cell membrane lipids to give rise to the occurrence of mutagenesis and cytotoxicity. It breaks down rapidly into water and oxygen, thus producing hydroxy radicals that can initiate lipid peroxidation and cause DNA damage in the body (Jan *et al*., 2013). Therefore, the eradication of hydrogen peroxide radical in order to prevent the body system from this radical is especially important. In this study, it was observed that the scavenging effect of the extracts is in the following order methanol > ethyl acetate > *n*-hexane extracts (Figures 7-9). This result is in agreement with the report of Jan *et al*. (2013) who showed the same order in the scavenging ability of their plant extracts on H_2O_2 radicals. The Significant linear correlations of *C. alata* leaf extracts showed that there were scavenging activities in the extracts and could generally be considered as a potent source of antioxidants since the trend in the behavior of the extracts in hydrogen peroxide radical scavenging activity assay was like that of vitamin C and quercetin (Figures 7-9). Therefore, it can be deduced from this study that the extracts of *C. alata* had the potential to eradicate hydrogen peroxide as the concentration of the extracts increases but the activity can be said to be low when compared to the reference antioxidants (vitamin C and quercetin).

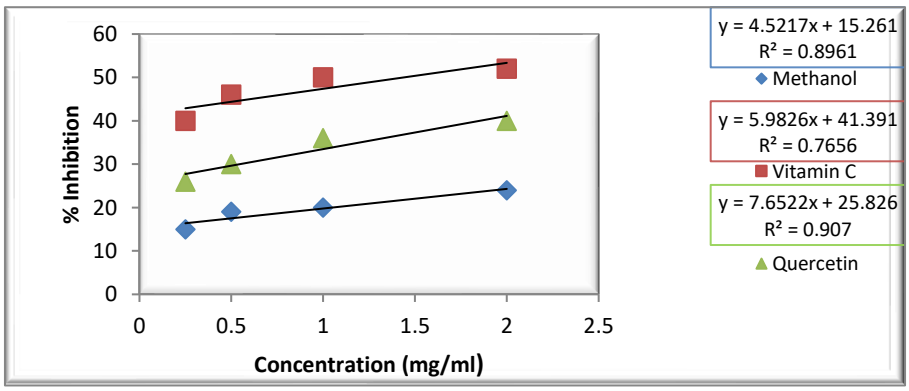

Figure 7: H_2O_2 scavenging activity of the methanol extract of *C. alata* leaf

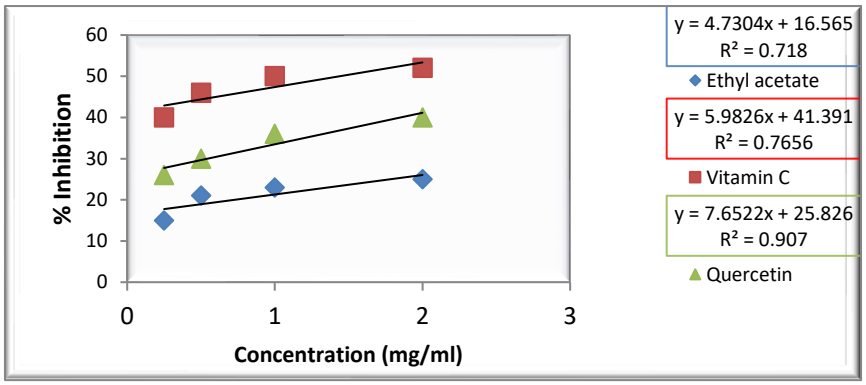

Figure 8: H_2O_2 scavenging activity of the ethyl acetate extract of *C. alata* leaf

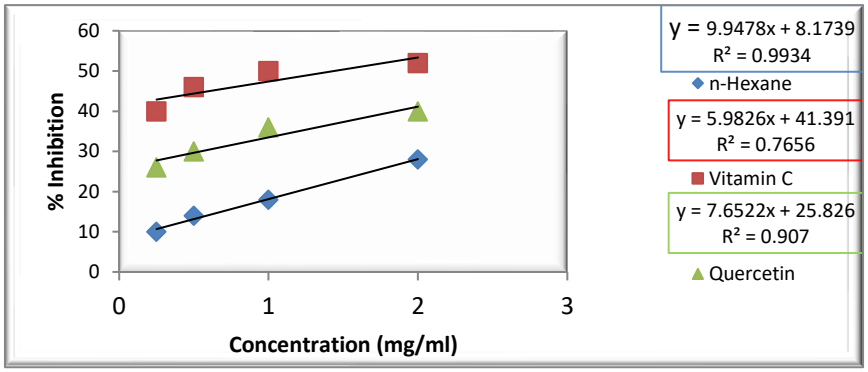

Figure 9: H_2O_2 scavenging activity of the *n*-hexane extract of *C. alata* leaf

The mutagenic capacity of free radicals is due to the direct interactions of hydroxy radicals with DNA, resulting in DNA breakdown and therefore plays an important role in cancer formation (Rahman *et al.*, 2015). The hydroxy radical is one of the most reactive oxygen species in living systems. Thus, the removal of OH radicals is particularly important for protecting biological systems. In this study, all extracts generally showed good hydroxy radical scavenging activity in a concentration-dependent manner (Figures 10-12). In general, *n*-hexane extract at 2.00 mg/L (15%) showed a much lower antioxidant potential than other extracts, as well as vitamin C (40%) and quercetin (35%). Here, we assumed that the antioxidant activity of the extracts (methanol and ethyl acetate) to quench hydroxy radicals might possibly relate to the inhibition of lipid peroxidation and acts as scavengers of active oxygen species by breaking free radical chains. Significant positive correlations were observed between the extracts and standards (Figures 10-12). Among the extracts, the ethyl acetate had a higher R^2 value (0.7577) than the other extracts (0.7216 and 0.7043). The activities of these plant extracts are indications of the presence phytochemicals that could be responsible for the observed antioxidant activities.

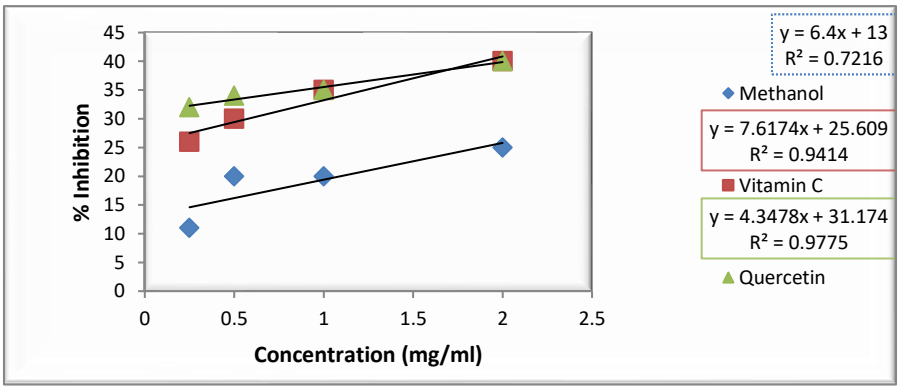

Figure 10: Hydroxy radical inhibitory activity of the methanol extract of *C. alata* leaf

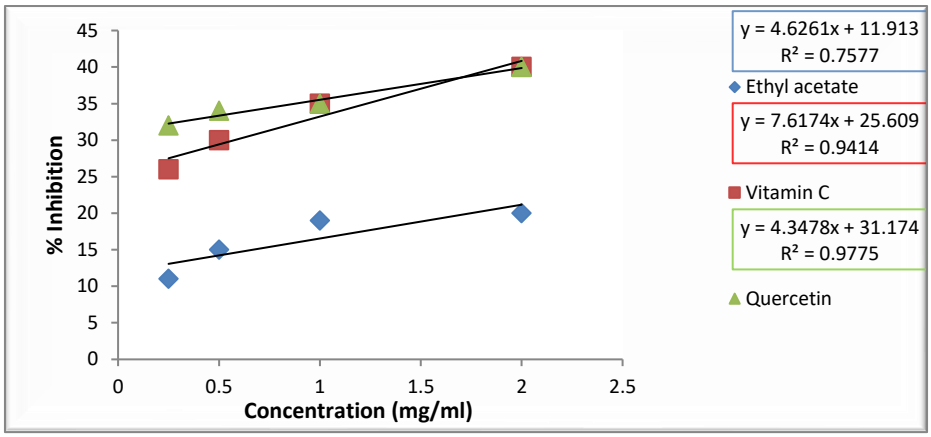

Figure 11: Hydroxy radical inhibitory activity of the ethyl acetate extract of *C. alata* leaf

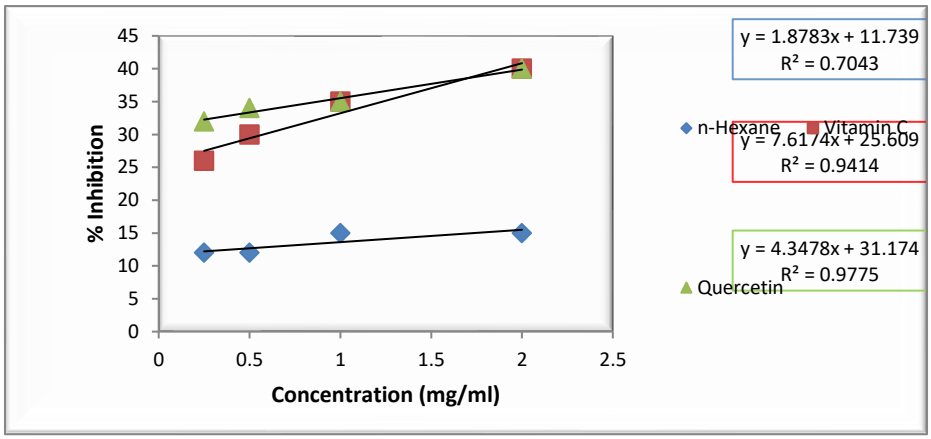

Figure 12: Hydroxy radical inhibitory activity of the *n*-hexane extract of *C. alata* leaf

CONCLUSION

In this study, the antioxidant activities and phytochemicals of the extracts of *Cassia alata* leaves were investigated. Phytochemical screening revealed that the methanol and ethyl acetate extracts contain more phytochemicals compared to the *n*-hexane extract. The result of the *in vitro* assays indicated that *C. alata* leaf is a potential source of natural antioxidants, which could help to prevent the progression of some diseases like cancers caused by free radicals. However, the components responsible for the anti-oxidative activity are currently unclear. This could be the focus of another study.

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