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Day time trend in the phytochemical constituents and antioxidant activity of *Azadirachta indica* leaves

Ogunbamowo P. O.* **and Awotedu O. L.**

Forestry Research Institute of Nigeria, PMB 5054, Jericho Hill Ibadan;
***Email:** *timilolo@yahoo.co.uk, awotedulekan@gmail.com*

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ABSTRACT

Background & Aim: Medicinal plants have been of immense therapeutic values to many communities, some traditional folklore places emphasis on collecting plants during the day for medicinal purpose. This study examined the effect of collection time on the phytochemical composition and antioxidant activity of *Azadirachta indica* leaves.

Experimental: Leaves samples were collected at six hour intervals between 6AM to 6PM, phytochemical quantification was done using standard methods; antioxidant assays were done after ethanol extraction via reducing power and nitric oxide scavenging assay (NO).

Results: Saponin concentration ranged from 1.08-1.24%, Tannin 0.22-0.31%, Steroid 0.08-0.14%, Glycoside 0.090-0.104%, Alkaloids 3.88-4.27%, Flavonoids 0.050-0.080% and 1.16-1.32% for Phenolics in the leaves of *A. indica* collected at different time of the day. The effect of time of sample collection on the phytochemical constituents shows that phytochemicals such as saponin, tannin, glycoside, alkaloids and phenolics are significantly ($p<0.05$) more concentrated in the leaves in the evening time (6PM) compared to other time period while steroid and flavonoid are also significantly more concentrated in the noon time (12PM). Reducing power assay shows that leave samples collected at 12PM exhibited the highest reducing power which is comparable with the samples collected at 6PM. The early morning samples showed the least reducing power while the standard antioxidant had higher reducing power than the three sample extracts. The highest percentage NO inhibition was observed in leave samples collected at 12PM (72.40%), but this is less than the ascorbic acid (standard antioxidant) which shows 84.47% inhibition. Analysis of the IC50 revealed that the afternoon sample (12PM) had the least value (23.29 µg/ml) thus, possessing better antioxidant capacity with respect to the NO scavenging; this is to a lower extent than the IC50 value (12.71 µg/ml) of the standard antioxidant.

Recommended applications/industries: From the foregoing, variation exists in daytime concentrations of phytochemical in A. indica leaves which may impact on its pharmacological actions.

1. Introduction

Since time immemorial, naturally obtained remedy have played a role in the development of human healthcare, according to an estimate by the World Health Organization, 80% of the populace in developing nations believe in indigenous based medicines which are mostly drawn from plants for their basic health care need. It is also noted that, no less than 25% of drugs obtained from plants, as well as many others that are chemically synthesized equivalents, created on model compounds extracted from plants are documented in modern pharmacopoeias (Bodeker *et al.*, 1997). Forest trees are loaded with an array of secondary metabolites such as phenols, flavonoids, terpenoids, alkaloids, tannins etc. (collectively called phytochemicals), some of which have been determined in-vitro to have therapeutic characteristics, thus, pharmacological based investigations have acknowledged the significance of medicinal plants as possible source of bioactive compounds (Biswas *et al.*, 2002) and phytochemicals present in medicinal plants could act as lead compounds in the discovery of antimicrobial substances (Ebi and Ofoefule, 2000; Cohen, 2000). Phytochemicals refer to chemical compounds that are naturally present in plants, they add to the flavour, colour and smells of plants while also forming a part of the natural defence system of the plant against diseases; their medicinal worth in prevention of diseases and ultimately human health are well documented (Okwu, 2004). They also serve as natural antioxidants which alleviate a number of degenerative diseases. Medicinal plants are rich in antioxidant compounds, and the potentials of such plants as antioxidants have been related to specific phytochemicals like flavonoids and phenols; and the therapeutic potential of plants are also correlated with their antioxidant potentials (Akinmalodun *et al.*, 2007; Eleazu *et al.*, 2011).

Azadirachta indica A. Juss. commonly known as Neem is a member of the Meliaceae family, it is indigenous to India and established in many of the tropical and subtropical countries including Nigeria. Neem has enormous medicinal potential and it is widely distributed throughout the world. The plant contains many biologically active substances including titerpenoids, carotenoids, ketones, flavonoids, alkaloids, and steroids, some of which had been previously reported (Krishnaiah *et al.*, 2009; Eleazu *et al.*, 2012; Vinoth *et al.*, 2012; Ndukwe *et al.*, 2013; Harry-Asobara and Samson, 2014; Madaki *et al.*, 2016). Neem have many valuable non-wood products such as bark, leaves, fruits, flowers, gum, seeds and oils derived from it than any other tree species, some of these products have been observed to possess antifungal, anti-allergenic, anti-inflammatory, nematicidal, insecticidal, antioxidant, larvicidal, spermicidal and other biological activities (Nahak and Sahu, 2010; Ogunleye, 2010; Enyi-Idoh *et al.*, 2012; Vinoth *et al.*, 2012; El-hawary *et al.*, 2013; Pandey *et al.*, 2014; Okoh *et al.*, 2015; Mohammed and Omer, 2015; Itelima *et al.*, 2016). The extracts from neem plant have also been reported to have termiticidal effect on wood termites (Sotannde *et al.*, 2011).

Previous studies have indicated that seasonal variation have an influence in the availability of active principles in medicinal plants as well as their therapeutic efficacy (Kpadonou Kpoviessi *et al.*, 2012; Castelo *et al.*, 2012; Asghari *et al.*, 2014; Soni *et al.*, 2015). Study by Ogunjinmi *et al*., (2014) demonstrated the effect of plant sample collection time on the phytochemical screening and antibacterial activities of *Chromolaena odorata* leaf extracts; day time disparity in the chemical constituents of essential oil from medicinal plants such as *Lantana camara* (Sousa *et al.*, 2010), *Pycnocycla spinose* (Asghari *et al*., 2014) and *Psidium guajava* (Silva *et al*., 2016), have also been reported. Some traditional folklore places emphasis on collecting plants during the day for medicinal purposes, it is believed that collecting medicinal plant's part for medicinal use in the evening is not advisable because there would be decrease in the medicinal efficacies of such plant's part (Ogunjinmi *et al.*, 2014). Whether this is a myth or fact remains to be seen. However, no study has documented the daytime variation in chemical composition of *A. indica* leaves; it is therefore necessary to establish a scientific basis for this belief and determine the best harvesting time for medicinal plant's part. It is against this background that this study assessed the daytime differences in the phytochemical composition and antioxidant activity of *A. indica* leaves.

2. Materials and Methods

2.1. Collection and extraction of plant samples

Sample collection and preparation was done at the Federal College of Forestry Ibadan. Leave samples were collected from the *A. indica* trees within the college premise at different time (6AM, 12PM, and 6PM) on the same day in October 2016, and labelled appropriately, a total of six (6) samples (duplicate for each collection time and combined together to represent each collection time) were collected. Sample was identified by a taxonomist at Forestry Research Institute of Nigeria. The leaves were rinsed with distilled water to eliminate dust and other unwanted materials and air dried for three (3) weeks. The dried leaf samples for each collection time was then pulverized using an electric blender, then 100g of the pulverized samples were macerated in 95% ethanol (200 ml) for five (5) days separately with constant daily stirring. The respective extracts of different collection time were filtered using Whatman filter paper No.1, and the filtrates concentrated under vacuum using a rotary evaporator at 40 °C and stored in preparation for the antioxidant assays (Aqil *et al.*, 2006).

2.2. Phytochemical analysis

Quantitative determination of phytochemicals such as Saponin, Steroids, Tannin, Glycosides, Alkaloids, Flavonoids and Phenolics in the ground samples were done according to the standard methods described below.

2.2.1. Determination of saponin

Saponin content was determined gravimetrically following the procedure previously used by Ezeonu and Ejikeme, (2016). 5g of pulverized sample for each collection time was extracted using 20% aqueous ethanol (100ml) for 4hrs in a water bath set to 55 ^{o}C with constant mixing, the solution was filtered to recover the residue which was then repeatedly re-extracted going through the previous process, the extracts obtained were combined and concentrated over water bath to 40 ml at 90 ^{o}C, then, diethyl ether (20 ml) was added in a separating funnel with vigorous shaking; the ether layer was dispose of, while the aqueous layer was retained after repeated purifications. Then, n-butanol (60 ml) was introduced, the mixture extracted twice using 10 ml NaCl (5 %). The NaCl layer was discarded while the residual mixture was further concentrated for 30 minutes in a water bath, the mixture decanted into a crucible and dried to a constant weight in an oven. % Saponin concentration was estimated using the formula:

$$\%\ Saponin = \frac{\text{Wt of Saponin}}{\text{Wt. of Sample}} \times 100$$

2.2.2. Determination of flavonoids

Quantitative flavonoid level was determined using the procedure described by Ejikeme *et al.*, (2014). Five grams of pulverized samples for each collection time was extracted with 50 ml of 80% aqueous methanol for 24hrs at ambient temperature, the mixture was filtered while the filtrate was discarded and the residue re-extracted thrice with 50 ml of ethanol; then the filtrate was combined and decanted into a crucible with the liquid evaporated to dryness in a water bath after which the crucible with its content was cooled in a desiccator and weighed to constant weight. The % flavonoid was calculated using the formula:

$$\%\ Flavonoid = \frac{\text{Wt of Flavonoid}}{\text{Wt.of Sample}} \times 100$$

2.2.3. Determination of phenol

The total phenols in the three samples was determined by the method previously used by Kim *et al.*, (2007) with little modifications. 10g of ground sample was treated with 30ml of 80% methanol in a 250ml beaker at 25°C in the dark to extract the phenolic. The mixture was transferred to warring blender and homogenized three times for an hour with successive addition of 30ml of 80% methanol. The homogenized mixture was then filtered using a Whatman No 1 filter paper into a volumetric flask of 100ml, then made up to mark with 80% methanol. 1ml of phenolic extract was added to 1ml of folin-ciocaltal reagent followed by incubation for 5mins at 22^{o}C and then addition of 5ml of 20% sodium carbonate. 0-10ppm of gallic acid standard solutions were prepared from 100ppm garlic acid standard and treated similarly like the samples and then read at 735nm on the spectrophotometer. % total phenolic was estimated using gallic acid as standard.

2.2.4. Determination of alkaloids

The gravimetric method (Harborne, 1973) was used in estimating the alkaloid contents of the samples. 2g of pulverized sample was extracted with 200 ml of 10% acetic acid prepared in ethanol for 4hrs, the resulting solution was filtered and the filtrate concentrated to a fourth of the original volume, this was then followed

by addition of concentrated NH_4OH (about 15 drops) to the extract until there is complete precipitation. The mixture was allowed to settle for 3hrs after which the supernatant was dispensed with and the precipitates washed using 20 ml of 0.1M NH_4OH, filtered, the residue dried and weighed. The % alkaloid was calculated as:

$$\% \text{ Alkaloid} = \frac{\text{Wt of Alkaloid}}{\text{Wt. of Sample}} \times 100$$

2.2.5. Determination of glycosides

The total glycoside level was determined using the procedure described by Onwuka, (2005). 10g of samples collected at different times were extracted with ethanol after which 10ml of extract was transferred using a pipette into a 250ml conical flask, then addition of 50ml of chloroform, with the mixture agitated on a vortex mixer for an hour. The resultant solution was separated by filtration into a fresh 100ml conical flask, where 10 ml of pyridine and 2 ml of 2% sodium nitro-prusside were introduced and well agitated for 10 minutes. The development of brownish yellow colouration was achieved by the addition of 3ml of 20% sodium hydroxide. Glycoside standards (0-5 mg/ml) were prepared from stock, and taken through the analytical procedure described above. The absorbance of the samples and those of standards were read at a wavelength of 510nm using a spectrophotometer. % glycoside of the samples was calculated from the concentration extrapolated from the standard curve absorbance against concentration.

2.2.6. Determination of tannins

The tannin content was determined according to the AOAC (1999) methods. Two grams of pulverized sample was weighed into a beaker, 100ml distilled water was introduced and the resulting mixture placed in water bath to boil for an hour with constant agitation to achieve uniform mixing, then, the mixture was separated by filtration into 100 ml volumetric flask using Whatman filter paper. Colour development was achieved through the addition of 5 ml of Folin-Denis reagent, 10 ml of saturated Na_2CO_3 and 5 ml of distilled water with 10ml of extract, after which the resultant solution was allowed to stand for 30 minutes at a temperature of 25 ^oC in a water bath with thorough shaking. Different concentrations of tannic acid (0-1mg/ml) was prepared from tannic acid stock and taken through the same procedures as the samples, then the absorbance of both samples and standards were taken at 700nm using spectrophotometer. Tannin amount was computed from the concentration value extrapolated from the calibration curve using the formula:

$$\% \, Tannin = \frac{\text{Concentration of sample} \times \text{Extract Volume} \times 100}{\text{Aliquot Volume} \times \text{Wt.of sample} \times 1000}$$

2.2.7. Determination of steroids

2 g of pulverized sample was transferred into a 100ml beaker, then, 20 ml of a mixture of chloroform and methanol in the ratio 2:1 was added and agitated for 30 minutes on a mechanical shaker; the mixture was then filtered using a Whatman No 1 filter paper into a new 100 ml beaker. The residue was repeatedly extracted for steroids with a mixture of chloroform/ethanol, filtered and the resultant filtrates combined. Then, 1 ml of the filtrate was measured into boiling tube, and alcoholic potassium hydroxide (5ml) was added with uniform mixing; the resultant solution was heated for 90 minutes in a water bath set at 40 °C, followed by cooling to room temperature and then, addition of 10 ml of petroleum ether and 5 ml of distilled water. The resultant mixture was placed on the water bath to evaporate to dryness, then 5ml of Burchard reagent was introduced to the residue and absorbance reading taken on spectrophotometer at 620nm. Standard steroid of concentrations (0-4mg/ml) were prepared from stock and taken through the same analytical process as the samples (Trease and Evans, 1989). % steroid was calculated using the formula:

$$\% \, Steroid = \frac{\text{Concentration of sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{Wt.of sample} \times 10000}$$

2.3. Determination of antioxidant activity

2.3.1. Determination reducing power

The principle behind the reducing power assay is centred on the conversion of Iron (III) (Fe^{3+}) to Iron (II) (Fe^{2+}) in the presence of solvent fractions (Navabi *et al*., 2008). The transformation to Fe^{2+} was observed by taking the absorbance reading of Perl's Prussian blue colour formed at 700 nm according to the method described by Farhoosh *et al.* (2007) with slight changes. 100µg/m l of the ethanolic extracts of plant samples collected at different time was reacted with 2 ml of 0.2M phosphate buffer (pH 6.6) and 10mg/ml potassium ferricyanide (2 ml), the mixture was incubated for 20 minutes at 50 ^oC and then, 2ml of 100ppm trichloroacetic acid was introduced into it. The

resulting mixture was separated by centrifugation at 3000 rpm for 10 min and the supernatant recovered, then, 2 ml of each supernatant layer was combined with 2ml of distilled water and 0.1% (w/v) freshly prepared ferric chloride (0.4 ml). The absorbance was measured on spectrophotometer at 700 nm after 10 minutes of reaction. The higher the absorbance, the better the reducing power.

2.3.2. Determination nitric oxide assay

The nitric oxide scavenging activity was evaluated in accordance with the procedure previously used by Okoh *et al.*, (2015), where sodium nitroprusside was used to generate nitric oxide radicals at physiological pH. 1 ml of neem leaf extracts of different harvest times prepared in variable concentrations (6.25-100 µg/ml) was added to sodium nitroprusside (10mM) in phosphate buffer (pH 7.4), this was followed by the incubation of the solution at 25°C for 2 hrs 30 min, then 1 ml of the incubated solution was measured into a fresh test tube followed by the addition of 1 ml of Griess'reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) and the absorbance was read at 546 nm. Ascorbic acid in same concentration range as the extracts and the control which is made up of equivalent amount of ethanol without the extract was taken through same procedure and the absorbance measured at same wavelength. The percentage inhibition of the nitric oxide radical by the extracts and the ascorbic acid (standard antioxidant) were computed with respect to the control using the formula:

$$\%\ NO\ Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

2.4. Data analysis

Data generated were analysed statistically using SPSS version 20 and MS Excel, results are shown as means±standard deviation of triplicate measurements; IC_{50} for each extract and standard was estimated from linear regression of the percentage inhibition and concentration data. Analysis of variance was used to test significant variations in the mean of the measured parameter between different collection times while LSD was used to separate mean differences; $p<0.05$ was considered significant.

3. Results and discussion

3.1. Trends in phytochemical composition

The result of the quantitative phytochemical composition of *A. indica* leaves collected at different time of the day is presented in Table 1. It shows that saponin concentration ranged from 1.08-1.24%, Tannin: 0.22-0.31%, Steroid: 0.08-0.14%, Glycoside: 0.090-0.104%, Alkaloids: 3.88-4.27%, Flavonoids: 0.050-0.080% and 1.16-1.32% for Phenolics in the leaves of *A. indica* collected at different time of the day. It is widely accepted that the therapeutic essence of plants is attributed to the existence of certain chemical substances possessing a definite physiological actions on living organisms, phytochemicals present in the leaves of *A. indica* are responsible for its diverse bioactivities reported by several authors, thus, the concentration of these compounds in the plant is of utmost importance. The concentration of phytochemicals in the plant at different time during the day was assessed in this study, the range of values for saponin obtained are comparable with the range of 0.54-1.00% reported in other studies (Krishnaiah *et al.*, 2009; Eleazu *et al.*, 2012; Ndukwe *et al.*, 2013; Ogbonna *et al.*, 2016), however, it is on a lower side compared to 6% reported by Enyi-Idoh *et al* (2012); the range of tannin concentration is within the range of 0.17-0.36% reported in previous studies (Eleazu *et al.*, 2012; Harry-Asobara and Samson, 2014; Enyi-Idoh *et al*., 2012; Ogbonna, *et al.*, 2016), which is quite lower than those reported by Ndukwe *et al.*, (2013) (1.16%), Pandey *et al*., (2014) (1.83%) and Krishnaiah *et al*., (2009) (9.1%). Alkaloid levels observed in this study compares favourably with the range of 1.38-4.00% reported in other studies for *A. indica* (Eleazu *et al.*, 2012; Harry-Asobara and Samson, 2014; Enyi-Idoh *et al*., 2012; Ogbonna *et al.*, 2016). It is much higher than those reported by Krishnaiah *et al.*, (2009) (0.52%) and Ndukwe *et al*., (2013) (0.85%), this may be as a result of difference in origin. Flavonoids concentration range in this study is significantly lower compared to the range of 0.43-0.43% previously reported (Krishnaiah *et al*., 2009; Ndukwe *et al.*, 2013; Harry-Asobara and Samson, 2014; Ogbonna *et al.*, 2016) and 5.33-7.5% range in the study of Enyi-Idoh *et al*., (2012), Eleazu *et al*., (2012) and Pandey *et al*., (2014), while phenolic concentration range is comparable to the findings of Pandey *et al*., (2014) and higher when related with other studies (Krishnaiah *et al.*, 2009; Ndukwe *et al.*,

2013; Harry-Asobara and Samson, 2014; Ogbonna *et al.*, 2016).

Table 1: Daytime variation in phytochemical composition (%) of A. indica leaves

Collection time	Saponin	Tannin	Steroid	Glycoside	Alkaloids	Flavonoids	Phenolics
6 AM	1.19±0.03^{a}	0.25±0.05^{a}	0.08±0.03^{a}	0.090±0.001^{a}	3.88±0.32^{a}	0.05±0.01^{a}	1.23±0.20^{a}
12 PM	1.08±0.06^{b}	0.22±0.10^{a}	0.14±0.02^{b}	0.096±0.001^{b}	4.02±0.25^{a}	0.08±0.02^{b}	1.16±0.16^{b}
6 PM	1.24±0.04^{a}	0.31±0.12^{b}	0.09±0.02^{a}	0.104±0.000^{c}	4.27±0.34^{b}	0.06±0.01^{c}	1.32±0.15^{c}

The observed differences in the phytochemical constituent in this study and in other studies may be a reflection of provenance, sample preparation and extraction process where different solvent have been demonstrated to possess greater extractive power than others (Nahak and Sahu, 2010). The effect of time of collection on the phytochemical constituents shows that certain phytochemicals such as saponin, tannin, glycoside, alkaloids and phenolics are significantly ($p<0.05$) more concentrated in the leaves of *A. indica* in the evening time (6PM) compared to other time period while steroid and flavonoid are also significantly more concentrated in the noon time (12PM) compared to other time period. Differences in the weather condition within these time period may account for the observed difference.

3.2. Trends in antioxidant activities

The antioxidant activity of *A. indica* leaves and essential oil extracts using various methods is widely reported (Ghimeray *et al.*, 2009; Nahak and Sahu, 2010; Okoh *et al.*, 2015) demonstrating its potential as a plant based antioxidant source. In order to determine whether time of sample collection may impact on pharmacological activities of medicinal plants, the daytime trend in antioxidant activity of *A. indica* was demonstrated using two in-vitro assays viz. Reducing power and Nitric oxide scavenging assay, where the variation in the antioxidant activity of *A. indica* sample extracts of different collection times were measured and related with Ascorbic acid (Vitamin C) a standard antioxidant, the results are presented in Figure 1 and 2.

The reducing power of a substance or compound refers to its capacity to serve as a reducing agent through the transfer of electrons; this may therefore serve as good pointer of its antioxidant activity (Koruthu *et al.*, 2011), based on the premise that the ability of certain antioxidants to reduce ferric ions signify their capacity to reduce free radicals and reactive oxygen species (Pinchuk *et al.*, 2012). Results from the reducing power assay shows that *A. indica* leave samples collected at 12PM showed the highest reducing power which is relatively comparable with the samples collected at 6PM. The early morning samples showed the significantly ($p<0.05$) least reducing power while the standard antioxidant had comparatively higher reducing power which is significant than the three sample extracts (Figure 1).

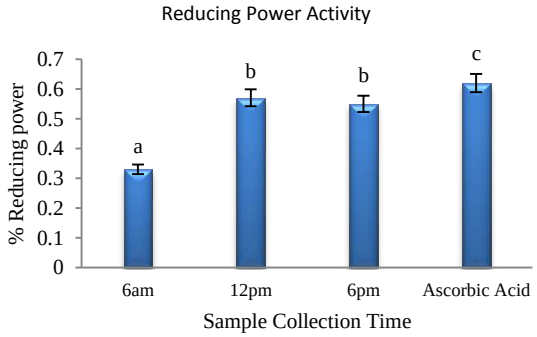

Figure 1: Daytime trend in reducing power activity. Bars with the same alphabets are not significantly different from each other at $p<0.05$.

Nitric oxide is an essential bio-regulatory molecule which possess some physiological actions some of which include platelet function, neural signal transduction and control of blood pressure (Jagetia *et al.*, 2014); however, it also exhibits toxic tendency when it reacts with oxygen and superoxide radicals, thus, excessive production of NO has been linked to several diseased state (Kelm, 1999). In the Nitric oxide scavenging assay, the result is presented as percentage inhibition of the NO radicals generated in the assay; a dose dependent inhibition was observed across the three extracts and standard. The highest percentage inhibition was observed in neem leave samples collected at 12PM, but this is less than the ascorbic acid (standard antioxidant) which shows 84.47% inhibition at the highest concentration used in this study. The maximum percentage inhibition of NO radicals by

plants collected in the morning and evening are 61.52% and 63.70% respectively (Figure 2).

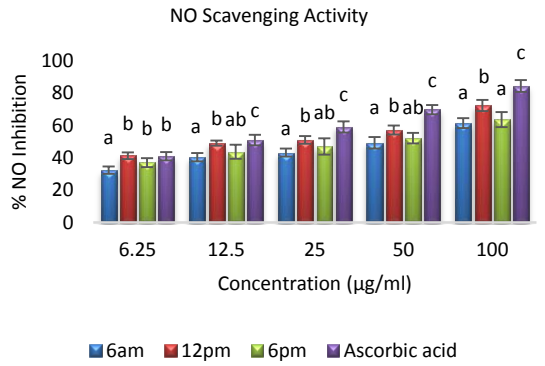

Figure 2: Daytime trend in nitric oxide scavenging effect of the extract of Neem leaves. Bars with the same alphabets are not significantly different from each other at $p<0.05$

Analysis of the IC_{50} (Figure 3) revealed that the afternoon sample had the lowest value (23.29 µg/ml) thus, possessing better antioxidant capacity with respect to the NO scavenging; this however, is lower than the IC_{50} value (12.71 µg/ml) of the standard antioxidant. Results from this study appears to suggests that neem samples collected at noon had better antioxidant activities than those collected at other time period, this could be that certain phytochemicals which are antioxidants may be concentrated in the leaves around this time period may be responsible for this activity.

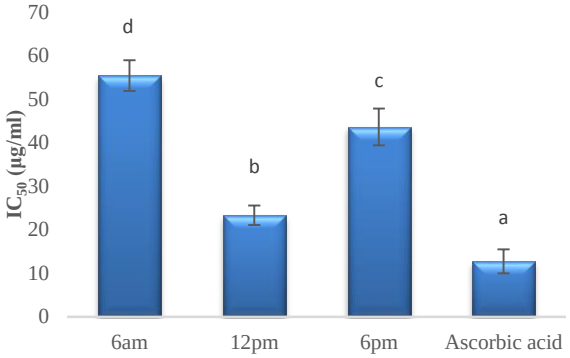

Figure 3: Comparative IC_{50} value of different sampling time and ascorbic acid. Bars with the same alphabets are not significantly different from each other at $p<0.05$

The variation in the pharmacological activity in terms of antioxidant effect of *A. indica* leaves collected at different time slightly mirrors the observation in the study by Ogunjinmi *et al.*, (2014) where the time of collection affected the antibacterial activity of *Chromolaena odorata* leaves; and certain bacterial strains were reported to be more sensitive to samples collected in the morning (7AM) and afternoon (4PM), however, in this study, evening samples (6PM) appears to show appreciable antioxidant activity compared with the morning samples, which is rather not surprising owing to the significant levels of certain phytochemicals in the plant around this time period.

4. Conclusion

The study has shown daytime variation in phytochemical constituents of *A. indica* leaves, daily trend in antioxidant activity suggests that samples collected at noon (12PM) may have better pharmacological activity which is closely followed by the evening (6PM) samples, though, further studies may be needed to substantiate this observation using other pharmacological actions both in-vitro and in-vivo while also considering seasonal variation and its pharmacological effect.

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