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Phytochemical profile and proximate composition of *Azadirachta indica* (A. Juss) harvested at different times of the day

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Abstract
The present work was carried out to investigate the effect of time of collection on the concentration of bioactive phytochemical constituents and proximate composition of *Azadirachta indica* leaves. The plant materials were collected in a day in the month of November at 6am, 9am, 12noon, 3pm and 6pm. phytochemical analysis of crude and methanol extracts, proximate parameters (moisture, protein, carbohydrate, fat and ash) were evaluated in the plant sample using the standard procedure of AOAC. The results from qualitative phytochemical analysis revealed the presence of saponins, alkaloids, tannins, phenols, flavonoids, terpenoids, triterpenoids, cardiac glycosides and reducing sugar in both the crude and methanol extract of *A. indica* leaf. The quantitative phytochemical estimations of the methanol extract showed highest value of saponins, total phenols and tannins at 12noon, flavonoids and alkaloids at 3pm while least values of saponins, total phenol and tannins were recorded at 6am. Highest amount of crude protein, ash and crude fiber were recorded in the morning. This result together with low crude fat in the morning could make the plant nutritionally good for collection before 9am. Our findings suggest that *A. indica* leaves could be harvested in the evening for medicinal purpose and best utilized by pharmaceutical industries.

Keywords: Phytochemical, medicinal, pharmaceutical, *Azadirachta indica*

1. Introduction
Azadirachta indica (A. Juss) commonly known as neem belongs to the family Meliaceae. It is a fast growing tree that can reach a height of 15-20m. It is an evergreen tree but may shed most of its leaves during severe drought. The tree has been introduced and established throughout the tropics and subtropics for its highly valued hardiness. Neem is considered to be a pharmacy in India where virtually every part of the plant is used medicinally. Leaf of neem trees are traditionally used to treat malaria, peptic ulcer and intestinal worm. Azadirachtin and other limonoids in neem plant extracts are active on malaria vector [1]. More than 140 compounds including alkaloids, flavonoids, triterpenoids, phenol compound, steroids, azadirachtin and nimbin have been isolated from different parts of neem [2].Various parts of *A. indica* have antiseptic, diuretic and purgative actions. They are also used to treat boils, pimples, eye diseases, hepatitis and rheumatism [3]. The act of using medicinal plants antedates human civilization and common medicine irrespective of technological development [4]. Medicinal plants are plants which contain bioactive substances that can be used for various therapeutic purposes [5].The medicinal properties of these plants could be attributed to secondary metabolites in them which have both stimulatory and inhibitory properties with physiological and biological actions in the human body [6,7]. The bioactive substances are found virtually in all parts of plant include saponins, tannins, flavonoids, steroids, alkaloids and phenols [8] which provide a wide range of functional properties. Several publications and literature have documented phytochemical profile of various medicinal plants globally [9, 10]. Environmental factors affect not only plant physiology (growth) but also influence secondary metabolites (bioactive ingredients) there in. Yadav and Kumar [11] reported that contents of secondary metabolites in plants is significantly affected by different environmental (abiotic) and diurnal variations. Several assumptions have been made by the traditional healers in Nigeria regarding the time and season for the collection of various parts of the medicinal plants. Traditionally, there is generally belief about the efficacy of the plant parts collected during a particular time of the day. Some plants are regarded as been potent traditional value drug when harvested in the morning (before sun rise), mid-day or evening (after sun set). Scientifically, several researchers have reported the effects of seasonal variation on secondary metabolites in different medicinal plants [12, 13, 14]. Data on diurnal variation of phytoconstituents of eight medicinal plants were documented by Anagha and Archarya [15],

There is dearth of information on the variation of bioactive secondary metabolites of medicinal plants collected at different time of the day in Nigeria. Hence, this work is a preliminary study on the phytochemical and proximate compositions of *Azadirachta indica* (Neem) leaves collected at different times of the day.

2. Materials and Methods

2.1 Collection of Plant Materials

A. indica leaves were collected in IworokoEkiti, Ekiti State in the month of November, 2017. The plant was identified at the herbarium of the Department of Plant Science and Biotechnology, Ekiti State University, Ado Ekiti. The leaves were collected at different time in a day starting from 6:00am, 9:00am, 12:00noon, 3:00pm and 6:00pm. The leaves were washed thoroughly using distilled water, air-dried for two weeks and then turned into powdered form using blender. The materials were kept in separate clean air tight containers at room temperature. The plant samples were taken to the Chemistry Laboratory, Chemistry Department of Afe Babalola University, Ado Ekiti, Ekiti State for Phytochemical and proximate analyses.

2.2 Phytochemical Screening

2.2.1 Crude Extraction of the Plant Sample

Twenty grams of the powdered sample was taken and dissolved in 100ml of distilled water and allowed to stay for 24hrs. Then, the content was filtered using No 1 Whatman filter paper and the extract was kept in an air tight bottle.

2.3Methanol Extraction of the Plant Sample

Ten grams of powdered leaf of the plan were extracted with 80% of methanol at 40-50 ^{0}C in soxhlet extractor until the extract was clear. The extract was evaporated to the pasty form and was stored in a refrigerator at 4 ^{0}C for further analysis.

2.4 Qualitative Analyses

2.4.1 Test for Alkaloids: A few drops of Wagner's reagent (Potassium iodine) were added to 2ml of the plant extract. The formation of reddish brown precipitate showed the presence of alkaloids [16].

2.4.2 Test for Saponins: The ability of saponins to produce frothing in solution was used as screening test for saponins. About 0.5g of each plant sample was shaken with 5ml of distilled water in a test tube. Frothing which persists on warming was taken as preliminary evidence for the presence of saponins [17].

2.4.3 Test for Tannins: Five grams of each portion of plant sample was stirred with 100ml of distilled water, filtered and a test drop of 0.1% of Ferric chloride reagent was added to the 20ml of filtrate. A blue black green or blue green or blue green precipitate was taken as evidence for the presence of tannins [18].

2.4.4 Test for Flavonoids: Five milliliters of dilute ammonia solution was added to 0.5g of plant sample, followed by the addition of concentrated H_2SO_4. A yellow colorations formed indicated the presence of flavonoid which disappeared later on standing [8].

2.4.5 Test for Cardiac Glycosides (Keller-Killiani test): The plant sample (0.2g) was dissolved in 2ml of glacial acetic acid containing a drop of ferric chloride solution. 1ml of concentrated H_2S0_4 was then added. A brown ring obtained at interface indicated the presence of deoxy-sugar characteristics of cardenolides. A violet ring appeared below the ring while in the acetic layer; a greenish ring formed just above the brown ring and gradually spread throughout this layer [18].

2.4.6 Test for Terpenoids: Five milliliters of crude extract of the plant sample was mixed with 2ml of chloroform and 3ml of concentrated H_2SO_4 were carefully added to form layer. A reddish brown coloration at the interface was seen, and this showed the presence of terpenoids.

2.4.7 Test for Steroids: Two milliliters of acetic anhydride was added to 0.2g of plant sample, with 2ml of concentrated H_2SO_4. There was a colour change from violet to blue or green in some of the samples, thus indicating the presence of steroids [19].

2.4.8 Test for Phenols: Finely ground sample (1 g) were soaked in 25ml of 2% of HCL for 1hour and then filtered through 10cm Whatman No. 1 Filter paper. 5ml of each plant extract was mixed with 1ml of 0.30% Ammonium thiocyanate solution and few drops of ferric chloride solution. A brownish yellow colour indicates the presence of phenol.

2.5 Quantitative Analyses

Further quantification of the phytochemical constituents in the samples was determined using the standard procedures of Harbone [19], Boham and Kocipal [20], Obadoni and Ochukwo [17].

2.6 Proximate Analyses

The proximate analyses of the samples were carried out according to the standard procedures of Association of Official Analytical Chemist [21].Moisture content was determined by heating 5g of the sample placed in a crucible inside an oven at temperature of 105 ^{0}C to a constant weight. Ash content was determined by heating 5 g of the plant sample in a crucible placed in a muffle furnace maintained at 450 ^{0}C. Crude protein was determined by multiplying the nitrogen content of the sample by 6.25 [22]. Crude fat was determined by extracting 2g sample in a soxhlet extractor apparatus with petroleum ether in an oven at 50 ^{0}C [23]. Crude fiber was determined by digesting 5 g of the sample with $H_2SO_4$and NaOH. The residue was placed into a crucible in a muffle furnace at about 550 ^{0}C for 5 hours. Carbohydrate was determined according to Onwuka [22]. Available carbohydrate was calculated as follows:

% Available carbohydrate = 100-(% moisture + % Ash + % protein + % fiber).

2.7 Determination of Mineral Element

The mineral constituents (phosphorus, calcium, magnesium, potassium, and sodium) in the samples were analyzed with the use of Atomic Absorption Spectrophotometer (AAS). One gramme of dried sample was weighed into crucible placed in muffle furnace at 550 ^{0}C for 1 hour. The ash sample was dissolved in hot 10% HCl and HNO_3 (ratio3: 1) and diluted to 100ml standard flask with distilled water. The solution was read with Atomic Absorption Spectrophotometer (AAS). The readings were calculated in milligram per 100gramme.

All data collected were subjected to statistical Analysis of Variance (ANOVA) and Duncan's Multiple Range Test was used as a follow up test to separate the means.

3. Results and Discussion

The results obtained in this study revealed the presence of medicinal active constituents in the crude and methanol extracts of *A. indica*. The phytochemical screening of the crude and methanol extract of *A. indica* showed that the leaf of the plant contained saponins, alkaloids, tannins, phenols, reducing sugar, cardiac glycosides, flavonoids, terpenoid and triterpenoids. Steroid and quinones were absent in the two plant extracts (Table1 and 2).

Table 1: Qualitative phytochemical analysis of crude extract of *A. indica* leaf

Phytochemical/Time	6am	9am	12noon	3pm	6pm
Saponins	++	+	++	+	++
Alkaloids	+	+	+	+	+
Tannins	+	+	++	+	+
Phenols	+	+	++	+	+
Flavonoids	+	+	+	+	+
Steroids	-	-	-	-	-
Terpenoids	+	+	+	+	+
Triterpenoids	+	+	+	+	+
Cardiac glycosides	+	+	+	++	++
Quinones	-	-		-	-
Reducing sugar	+	+	+	+	+

Table 2: Qualitative phytochemical analysis of methanol extract of *A. indica* leaf

Phytochemical/time	6am	9am	12noon	3pm	6pm
Saponins	+	+	++	+	++
Alkaloids	+	+	+	+	+
Tannins	+	+	++	+	+
Phenols	+	+	++	+	+
Flavonoids	+	+	+	+	+
Steroids	-	-	-	-	-
Terpenoids	+	+	+	+	+
Triterpenoids	+	+	+	+	+
Cardiac glycosides	+	+	+	++	++
Quinones	-	-		-	-
Reducing sugar	+	+	+	+	+

+ indicates present, ++ indicates abundantly present, - indicates absent

Also, the quantitative estimation (mg/100g) of phytochemical constituents of *A. indica* was presented in Table 3. It is evident from this table that alkaloids concentration (mg/100g) during different time of the day (6am-6pm) range from 0.10-0.19, saponins from 0.10-0.14, total phenol varied from 4.95-6.47 and flavonoids from 12.87-14.28.

Table 3: Quantitative phytochemical estimation of methanol extract of *A. indica* leaf (mg/100g)

Parameter/time of collection	Alkaloids	Saponins	Total Phenol	Tannins	Flavonoids
6am	0.16±0.02c	0.07±0.01c	4.95±0.03d	2.47±0.05c	14.14±0.10a
9am	0.18±0.02b	0.09±0.01d	5.72±0.06c	3.10±0.05b	13.34±0.06b
12noon	0.14±0.01d	0.14±0.02a	6.47±0.09a	4.18±0.07a	14.11±0.09a
3pm	0.19±0.02a	0.10±0.00c	5.89±0.08c	3.31±0.06b	14.28±0.06a
6pm	0.10±0.02e	0.12±0.15b	6.09±0.03b	3.35±0.55b	12.87±0.10c

Note: Means with the same letter(s) along the column are not significantly different from each other

The percentage proximate yield of *A. indica* leaf between the hour of 6am and 6pm revealed that data obtained for moisture content range from 11.30-12.31, crude protein (17.18-20.98), crude fat (2.15-2.70), ash content (8.17-9.11), crude fiber (4.50-4.72) and carbohydrate (50.38-56.15).

Table 4: Percentage proximate estimation of *A. indica* leaf

Parameter/time of collection	Moisture content	Crude Protein	Crude Fat	Ash content	Crude Fiber	Carbohydrate
6am	12.31±0.12a	20.98±0.20a	2.15±0.08c	9.11±0.42a	4.72±0.06a	50.38±0.10c
9am	11.30±0.30b	18.98±0.01b	2.54±0.11a	9.07±0.01a	4.55±0.04b	53.62±0.12d
12noon	11.36±0.05b	18.79±0.06b	2.70±0.28a	8.44±0.28b	4.65±0.04a	54.07±0.03c
3pm	11.33±0.05b	17.18±0.06c	2.43±0.08b	8.40±0.42b	4.65±0.04a	56.15±0.07a
6pm	11.39±0.01b	18.20±0.06b	2.42±0.03b	8.17±0.14b	4.50±0.03b	55.32±0.06b

Several authors and different literatures have revealed that a number of botanicals contained various phytoconstituents like alkaloids, saponins, steroids and flavonoids [24, 25, 10, 26] which confirm in them antioxidant and antimicrobial properties [27]. Several beneficial health effects that have been attributed to *A. indica* plant may be as a result of their rich bioactive phytochemicals. Ashien [28] reported the presence of alkaloids, reducing sugar and some cardiac glycosides in the water and methanol extracts of dry neem leaves. The results of this study are in agreement with the phytochemical results of neem extracts published by Prashanth and Krishnaiah [29]. Qualitative estimation of alkaloids showed that alkaloids concentration was highest for 3pm and least for 6pm. Statistical analyses at 5% level of probability (DMRT) revealed that there were significant differences among the various results obtained at 6am, 9am, 12noon, 3pm and 6pm. The saponins concentration varied from 6 in the morning to 6 in the evening. The highest concentration was obtained at noon while the least value was recorded for 6am. However, when compared the values statistically ($P<0.05$) the results revealed that there was significant difference among the various concentration of saponins at different times of the day. Flavonoids recorded in this study had the highest concentration at 3pm and least at 6pm.

The values obtained when compared statistically at 6am and 3pm showed no significant difference in the two concentrations. However, there were significant differences when compared various flavonoids concentrations at 9am, 12noon and 6pm. The highest value of tannins was obtained at 12noon and the least at 6 in the morning. Statistical analyses of the results showed that there were significant differences among the various concentrations of tannins at 6am, 9am and 12noon. However, significant difference did not exist between values at 9am, 3pm and 6pm.

A. indica leaf had highest total phenol concentration at 12noon and least value at 6am.The results differ significantly when compared statistically at 5% level of probability (DMRT).

The results obtained at different times of the day revealed that highest concentration of phytochemicals were obtained between the hour of 12noon and 3pm when the sun intensity is usually high. Time is one of the major factors that influence the efficacy of medicinal plant. This can be seasonal, fortnight and day variation (Diurnal variation). Time of collection and harvesting of plant material also play important role in the quality of drug [15]. They documented significant differences in the amount of phytochemical contents and therapeutic activity of *Ocimum Gratissimum*, *Rosa damascene* and *Crocus sativum* as a result of diurnal variation. Diurnal fluctuations of the major alkaloid concentration in latex of poppy *Papaver somniferum* were investigated. The results are not reflections of enzymatic processes but the result of water transport between the laticifers and the surrounding vascular tissue [30]. Walker [31] reported that the herbal constituents of plants may vary depending on the harvest seasons couple with other factors.

It could be inferred from the above information that the high concentration of these phytochemical in the leaf of *A. indica* between the hour of 12noon and 3pm might be as a result of high intensity of sunshine. Sezai *et al*. [32] reported that stronger sunlight for a longer duration induced and enhanced the biosynthesis of phenolic. Stengele and Stanlis [33] reported that increase in temperature inhibits plant growth more than photosynthesis and more of the carbon fixed is made available for production of these secondary metabolites. Singh and Dhawan [34] equally reported that an increase in temperature stress brings about increase in production of secondary metabolites.

The highest moisture content was obtained from the leaves which were harvested at 6am and the lowest from the leaves harvested at 3pm. Statistical analyses however revealed that the percentage moisture content at different time of the day were not significantly different from one another except at 6am. Low moisture content obtained in the plant sample will help its shelf life. The percentage ash content of the investigated leaves showed the best result at 6am and the least value at 6pm in the evening. The percentage ash content of the leaves was not significantly different at 6am and 9am, 12noon and 3pm when compared statistically. Ash content is an indication of the level of inorganic minerals and organic matter present in the leaf. The result of crude fiber is similar to that of ash content with highest values at 6am and lowest value at 6pm.

The percentage protein yield of the leaf studied gave highest value at 6am and least at 3pm. Significant increase in carbohydrate was observed between 6am and 12noon. The results of the proximate composition support the collection of this plant in the morning. Highest value of crude protein, ash content, crude fiber and low crude fat in the morning is also an added advantage since consumption of food with low fat is advisable for human health. The amount of carbohydrate in the leaf in the morning (6am) is still moderate and could provide energy for the consumers.

4. Conclusion

It could be suggested from the above results that the plant leaves should be harvested in the morning if needed for nutritional purpose. The results of our findings equally support the collection of this plant leaves in the afternoon (12noon-3pm) when the secondary metabolites concentrations are highest. The results could serve as guide to pharmaceutical industries on appropriate time of harvesting of the plant for drug. However, it would be wrong to draw a conclusive perception about the efficacy of this plant without taking into consideration other specific environmental conditions.

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