

COMPARATIVE ANALYSIS ACTIVE INGREDIENTS IN NEEM (*Azadirachta indica*. L) LEAVES FOR DOSAGE REGULATION IN HERBAL PRODUCTS ADMINISTRATION

ABSTRACT

Plants used for treatment of diverse ailments primitively are concocted and used indiscriminately. Questionnaires were administered to 250 herbal users and dealers to establish the respective local herbal applications with regards to Mango leaves (*Mangifera indica*, Pawpaw (*Carica papaya*) and neem (*Azadirachta indica*) leaves. Neem leaves (*Azadirachta indica*) was endorsed for medicinal treatment of malaria fever. The leaves were plucked at appropriate peak period the day when oil and moisture contents high. The peak period of contents was found to occurred between 8am-10am on a very sunny days, 10am to 12 on cool days, and 3:00pm to 5:00pm on cloudy days. Aqueous extract after maceration from Neem leaves was subjected to both qualitative and quantitative analysis which revealed available phytochemicals to include; saponin (34.89mg/g), tannin (31.715mg/g), flavonoid (31.835mg/g), phenol (43.59mg/g), terpernoid (14.585mg/g), cardiac glycosides (39.335mg/g), steroid(16.185mg/g) and alkaloid (28.76mg/g). These values differ significantly to recommended oral dosage formulation human consumption: Saponin (1.433ml), Tannin (1.418ml), Flavonoid (13.91ml), Phenol (2.29ml), Terpernoid (8.23ml), Cardiac Glycosides (0.003177ml), Steroid (0.62mg/g). Consequently, local consumption of neem as herbal resource should be regulated to avoid abuse and long or short-term effects of drug contents in neem as local herbs.

Words: Phytochemicals, Calibration, Dosage, Peak periods, Herbal, Concocted

1.0 INTRODUCTION

The use of herbs for medicinal purpose is indigenous but the indiscriminate usage of herbal products by the people had become habitual in some localities because of their believes. This had become worrisome in recent years as people engage in haulage and hawking of diverse mixtures of herbal leaves, barks of trees and roots as well as some fruits without due consideration of the dosage and the long-term effects of the contents and its accumulation in the body system. Medicinal plants are used by humans to treat sickness (Sarbani (2019). Demand for medicinal herbs is increasing quickly, owing mostly to the negative side effects of synthetic chemical medicines, believed ineffectiveness of drugs in some cases, poverty lack of purchasing power, ignorance and addiction to available options. The global demand for natural ingredients encourages the cultivation of therapeutic crops on a local scale while regulated and sustainable collection of wild plants have not been given serious consideration. Such efforts have the potential to expand rural employment in developing nations, promote global commerce, and improve the lives of millions of people (Anita, 2004). The therapeutic value of plants is derived from chemical compounds that create physiological functions in the human body. The most important of these plant bioactive components are alkaloids, tannins, flavonoids and phenolic compound (Hill 1985).

Plants are good source of available and low-cost medication. Medicinal plants are therapeutically beneficial yet have little or no adverse effects (Odugbemi and Akinsulire, 2006). Plants and their mechanisms, such as roots, stems, barks, leaves, berries, fruits, seeds, and exudates, comprise an essential significant component of the medications employed in the medical system due to their low side effects and low cost. Medicinal plant extract and plant products reveals that some of those plants are important sources of antibiotics. Natural resources are widely acknowledged to be the source of many pharmacologically active medications, including medicinal plants. Nigeria is endowed with a vast array of plant species. This heritage provides for her people's food, medicine, and other necessities (Odugbemi and Akinsulire, 2006).

Some of the available medicinal plants parts engaged for use include; Pawpaw leaves, Mango leaves, Lemongrass, Chamomile, Eucalyptus, Aloe Vera, Scent Leaf, Butterleaf, Neem, Gingko, Teatree, Lavender, Moringa, Candle Plant, Wild Lettuce, Mint leaves, Water leave, Avocado leaves (Monier 2016). These plants are administered in divers forms singly and at times in selected combinations.

Azadirachta indica (Neem) is a tree that is evergreen. It is a member of the Meliaceae family, it plays an important role in health-promoting effect because it is a rich source of antioxidant. It has a complex of distinct composition such as Nimbin, Nimbidine, Nimbolide and Limonoids and these ingredients play a part in the treatment of diseases. Quercetin and β -sitosterol became the first polyphenols Flavonoids isolated from fresh neem leaves and considered to have antifungal and antibacterial function (Govindachari et.al, 1998). Neem has various biological and pharmacological effects such as antibacterial, antifungal, anti-inflammatory, antibiotics, antigastric ulcer antitumour activities, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, antimalarial (Bandyopadhyay et al., 2004). However, the qualitative analysis and application of this findings has not been put into use as local applications evolves around modulation of multiple constituents of different parts of medicinal plants concocted and used indiscriminately. It is enthused with a mindset that it can cure all forms of sickness as 'Gbogbonise' in some local settings in the South-West zone of Nigeria. Therefore, the objective of this work is to investigate evaluate the usage of neem leave extract in relevance to recommended dosage for human consumption.

2.0 MATERIALS AND METHODS

2.1 Preliminary Investigations

Questionnaires were prepared to investigate the most widely sourced medicinal leaves used for treatment of most common ailment (Malaria) in the local areas of Epe town suburb of Lagos State Nigeria. Suggested leaves investigated were Mango. Pawpaw and Neem leaves. The most recommended by the data analysis using five hedonic scale was chosen for the purpose of this research. The time appropriate for the collecting the leaves was also investigated using oil-moisture analysis of the leaves while on the stalk to obtain peak period of content hourly, between the hours of 8:00 am and 5:00 pm to obtain periods of maximum oil-water percent.

2.2 Sample Preparations

Fresh leaves free of contaminant and perforations were collected during the peak hours of the day between 10am-12pm when oil and moisture contents were at high percentage using an oil and

moisture content analyser. The freshly collected neem leaves was dried at room temperature $25\pm 2^{\circ}\text{C}$ until it was bone dried and stored in a desiccator in preparation for extraction (Biswas .et al, 2002). About 500g of the dried leaves was weighed and macerated to break cell wall to release soluble phytochemicals when dissolved. The macerated sample was soaked in 2000ml (98% Laboratory grade) ethanol to maintain a liquor ratio of 4:1 volume to weight (Handa et al., 2008). The media is frequently agitated every 3 hours at room temperature for a period of three days until the soluble matter properly dissolved. Lowering particle size increases surface contact between samples and extraction solvents. The extract from the macerated neem was collected by filtration using Whatman filter paper and subjected to further analysis, A sample of the fresh Neem leaves and the bone dried samples are shown in plate 1:



Plate 1a: freshly collected neem leave

Plate 1b: Bone dried Neem leaves samples

2.4 Analysis of phytochemical contents

Qualitative analysis was carried out to ascertain the presence of the different phytochemical compounds contained in the extract. The extracts were subjected to thin-layer and paper chromatographic procedures at the laboratory. This was done in order to separate the components into individual compounds for appropriate identification of all components of the extracts. Preliminary phytochemical analysis of the extracts was carried out with reference to the standard methods (Sofowora (1998)).

2.4.1 Estimation of Alkaloids

Total alkaloid was measured using methods as described by (Fazel *et al.*, 2008) with slight modification. 1ml plant extract, 5 ml pH 4.7 phosphate Buffer was added and 5 ml BCG solution and the mixture were shake with 4 ml of chloroform. The extracts were collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without extract. Atropine is used as a standard (using calibration curve) and compared the assay with Atropine equivalents.

Preparation of Standard Curve Accurately measure aliquots (0.4, 0.6, 0.8, 1 and 1.2 mL) of atropine standard solution and transfer each to different separatory funnels. Add 5 mL pH 4.7 phosphate buffer and 5 mL BCG solution. Shake mixture with 1, 2, 3 and 4 mL of chloroform. The extracts were collected in a 10 mL volumetric flask and then diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without atropine.

2.4.2 Estimation of Steroids

Total steroid was measured using methods as described by (Salomi *et al.*, 2019) with slight modification. 1ml of test extract of steroid solution was transferred into 10ml volumetric flasks, Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70 ± 2 °C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank. Total steroids in extracts was expressed in terms of cholesterol equivalents (mg of CHO/g of extract).

2.4.3 Estimation of Flavonoids

Aluminum chloride colorimetric method (Chang *et al.*, 2002) with some modifications was used to determine flavonoid content. Plant extract (1mL) in methanol was mixed with 1ml of methanol, 0.5 mL aluminum chloride (1.2%) and 0.5 mL potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature; then the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid content is expressed in terms of quercetin equivalent (mg g⁻¹ of extracted compound).

2.4.4 Estimation of Total Phenols

The Follins method described by (Pearson 1976) was used to determine the phenol content. 1ml of the extract was placed in a test tube, 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO₃ aqueous solution were added. The samples were thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was determined using spectrophotometer at wave length = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of gallic acid equivalent expressed in terms of (mg of GA/g of extract).

2.4.5 Estimation of Tannins

Tannin content was determined by the Folin-Denis colorimetric method described by Kirk and Sawyer (1998). 5g sample was dispersed in 50mls of distilled water and shaken. The mixture was allowed to stand for 30min at 28°C before it was filtered through Whatman No. 42 grade of filter paper. 2ml of the extract was dispersed into a 50ml volumetric flask. Similarly, 2ml standard tannin solution (tannic acid) and 2ml of distilled water were put in separate volumetric flasks to serve as standard and reagent was added to each of the flask and the 2.5ml of saturated Na₂CO₃ solution

added. The content of each flask was made up to 50ml with distilled water and allowed to incubate at 28°C for 90 min. Their respective absorbance was measured in a spectrophotometer at 765nm using the reagent blank to calibrate the instrument at zero.

2.4.6 DNS reducing sugar assay

This assay is based on the protocol described by Hussain *et al.*, (2016) with slight modification. 1ml of the plant samples was taken and 3ml of DNSA was added and boiled for 10min and absorbance was taken at 540nm. Glucose was used as standard (100mg/ml).

DNS reagent was prepared by mixing 1.6 g NaOH and 1.0 g dinitro salicylic acid (Sigma) in 70 mL dH₂O and the mixture was heated in boiling water to dissolve. Once dissolved, 3.0 g Na₂K tartrate (Sigma) was added to the solution and swirled until dissolved followed by further addition of H₂O to make up to 100 mL. The reagent was stored dark at room temperature. 1ml of the plant samples is taken and 3ml of DNSA was added and boiled for 10min and absorbance was taken at 540nm. Glucose was used as standard (100mg/ml).

2.4.7 In vitro Antioxidant Assays Reducing power assay (Oyaizu, 1986)

The reducing power was determined according to the method with slight modifications. Reaction was carried out in a mixture containing 1 ml of sample (25-100 µg/ml), 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1%, w/v potassium ferro cyanate [K₃Fe (CN)₆] by incubating at 50°C for 20 min. After addition of 2.5 ml trichloroacetic acid (10%, w/v), the mixture was centrifuged at 5000rpm for 10 min. The upper layer (5 ml) was mixed with 0.5 ml of fresh FeCl₃ (0.1%, w/v), and the absorbance at 700 nm was measured against a blank. Gallic acid was used as the control.

2.4.8 DPPH radical scavenging assay (Shimada *et al.*,1992).

Briefly 0.1 mM solution of DPPH in ethanol was prepared; 1ml of the solution was added to 1 ml of extract in water at different concentrations (25-100 µg/ml). The mixture was vigorously shaken and afterwards allowed to stand at room temperature for 30 min to settle. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer. When of the reaction mixture was subjected to Lower absorbance, it indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using equation 1;

$$\text{DPPH Scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \dots\dots\dots(1)$$

Where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the standard sample or extract. The IC₅₀ value represented the concentration of the compounds that caused 50% inhibition of DPPH radical formation.

2.4.10 Hydroxyl radical scavenging assay (Zhang *et al.*, 2008)

The mixture containing 1 ml of sample (0.1–0.5 mg/ml), 1 ml of 9 mM FeSO₄ and 1 ml of 0.3% H₂O₂ in 0.5 ml of 9 mM salicylic acid–ethanol solutions was shaken vigorously and incubated at 37°C for 30 min. Then, the absorbance of the reaction mixture was determined at 510 nm. Gallic Acid (GA) was used as the positive control. The hydroxyl radical scavenging activity was calculated by equation 2;

$$\text{Scavenging Activity (\%)} = \left[1 - \frac{A_1 - A_2}{A_0} \right] \times 100 \dots\dots\dots(2)$$

Where A_0 is the absorbance of the control (ethanol instead of sample); A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (salicylic acid– ethanol solution instead of FeSO_4 and H_2O_2 solutions). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of hydroxyl radical formation.

Superoxide radical scavenging assay (Zang et al., 2008)

Reaction was carried out in a mixture containing 4.5 ml of 50 mM Tris–HCl buffer (pH 8.2), 0.4 ml of 25 mM pyrogallol solution and 1 ml of sample (0.5–5 mg/mL) by incubating at 25°C for 5 min. Finally, 1 ml of 8 mM HCl solution was dripped into the mixture promptly to stop further reaction. The absorbance of the mixture was measured at 420 nm frequency. Gallic Acid was used as the positive control. The superoxide radical scavenging activity was valuated using equation 3;

$$\text{Scavenging activity (\%)} = \left[1 - \frac{A_1 - A_2}{A_1} \times 100 \right] \dots\dots\dots (3)$$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (Tris–HCl buffer instead of pyrogallol solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of superoxide radical formation.

2.4.11 Hydrogen peroxide radical scavenging assay (Sarbani, 2019)

Reaction mixture containing 1 ml of sample (0.1– 2 mg/mL), 2.4 ml of phosphate buffer (0.1 M, pH 7.4) and 0.6 ml of H_2O_2 solution (40 mM) was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230nm. Gallic Acid was used as the positive control. The H_2O_2 scavenging activity was calculated as follows

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (phosphate buffer instead of H_2O_2 solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of H_2O_2 . It is calculated by equation 4;

$$\text{Scavenging activity (\%)} = \left[1 - \frac{A_1 - A_2}{A_0} \times 100 \right] \dots\dots\dots (4)$$

2.4.12 β -carotene linoleate bleaching assay (Cheung et al.,2003)

In the assay, linoleic acid produces hydroperoxides (ROS), and this oxidizes the β - carotene at 50°C. The presence of antioxidants in the extract will minimize the oxidation of β -carotene by hydroperoxides. Hydroperoxides formed in this system will be inactivated by antioxidants from extracts. Gallic acid was used as the standard. B- Carotene (0.2 mg in 1 ml of chloroform), linoleic acid (0.02 ml), and Tween 80 (0.2 ml) were transferred into a round-bottomed flask. Chloroform was removed at room temperature using a rotary evaporator. Following evaporation, 50 ml of distilled water was added to the mixture and shaken vigorously to form an

emulsion. Two milliliters of aliquots of the emulsion was pipetted into test tubes containing of mushroom extracts (different concentration of 20–100 $\mu\text{g}/\text{ml}$) and immediately placed in a water bath at 50°C. The absorbance was read at 20 min intervals for 2 h at 470 nm using UV-vis spectrophotometer. The antioxidant activity (I) was expressed as a percent of inhibition relative to the control, using equation 5 below;

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \dots\dots\dots (5)$$

Where A_c and A_s represent the bleaching rates of β -carotene without and with the addition of antioxidant, respectively.

2.4.12 Lipid peroxidation Assay (Abdullah *et al.*, 2010)

The reaction mixture contained 1 mL of fowl egg yolk emulsified with phosphate buffer (pH 7.4) to obtain a final concentration of 25 g/l, sample (different concentration of 20–100 μ g/ml), and 100 μ L of 1000 μ M FeCl₂. The mixture was incubated at 37°C for 1 h before being treated with 0.5 ml of freshly prepared 15% trichloroacetic acid (TCA) and 1.0 ml of 1% thiobarbituric acid (TBA). The reaction tubes were further incubated in boiling water bath for 10 min. Once cooled to room temperature, the tubes were centrifuged at 3500 rpm for 10 min to remove precipitated protein. The absorbance at 532 nm was determined spectrophotometrically. Gallic acid was used as positive control. The percentage inhibition was calculated from equation 6;

$$\text{Inhibition (\%)} = \frac{A_b - A_s}{A_b} \times 100 \dots\dots\dots(6)$$

Where A_b is the absorbance of the blank without the extract or ascorbic acid and A_s is the absorbance in the presence of the extract or gallic acid

2.4.13 Inhibition percentage (Griess Reagent)

Sodium nitroprusside (10mM) in phosphate buffer saline was mixed with different concentrations (25-100 μ g/ml) of each extract, and incubated for 30min, after incubation period, 0.5ml of Griess reagent (1% sulfanilamide, 2% H₂PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. Absorbance was taken at 550nm and ascorbic acid was used as standard. IC₅₀ which is an inhibitory concentration of each extract required to reduce 50% of nitric oxide formation was determined. The same reaction mixture without the extract but equivalent amount of methylated spirit was used as control. It is calculated using equation 7;

$$\text{Nitric oxide (inhibition \%)} = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \times 100 \dots\dots\dots(7)$$

3.0 RESULTS AND DISCUSSION

3.1 PRELIMINARY INVESTIGATIONS

Questionnaires distributed to local merchants and users of herbs was analysed to investigate three medicinal leaves commonly taken for treatment of ailment such as malaria in EPE, suburban areas of Lagos State. The neem leaf was adjudged widely used for the treatment of malaria sickness either singly or along other recipes. The investigation showing the distribution of opinion poll on the uses of neem leaves choice and applications as shown in figure 1:

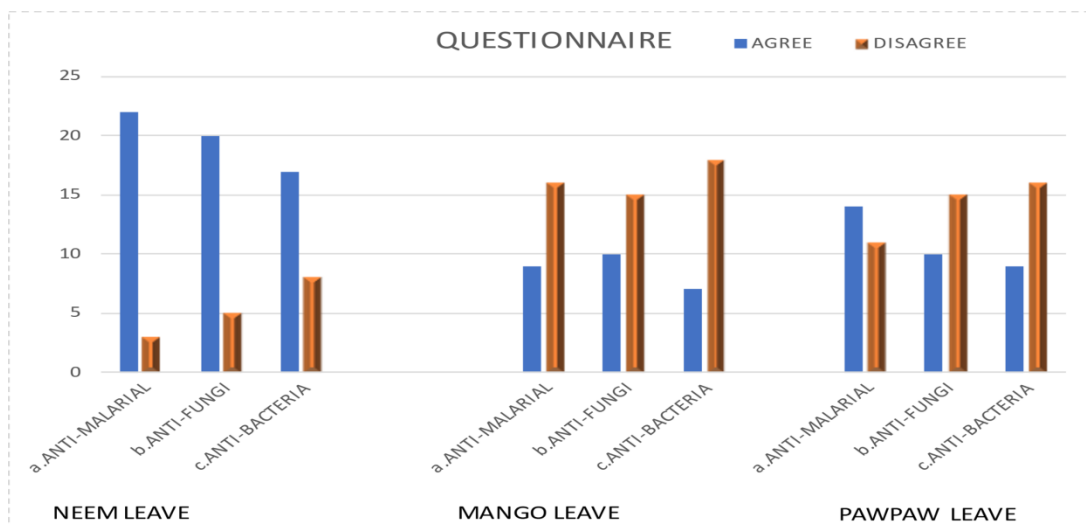


Fig 1: Analysis of questionnaire distribution

3.2 Oil and Moisture Content Analysis of Sampled leaves

The oil-moisture detector is used to analyse content of the leaves while still on the stalk hourly for five days between 8:00 am - 5:00 pm to determine the best time for sample collection. This occurred when the oil and water content recorded are practically at the peak. It was observed that the trend of oil and water movement along the plant morphology followed the same pattern of variation. highest and almost at the same level. In this regard, the right time for the harvest of leaves were found to be between 8am and 10am on early sun rise day, and on a fair weather day between 10 am and 12:00 Noon but on a cool days perhaps with rain, ideal time was between 3pm to 5pm as shown in Figure 2 Figure 3 and Figure 4 respectively:

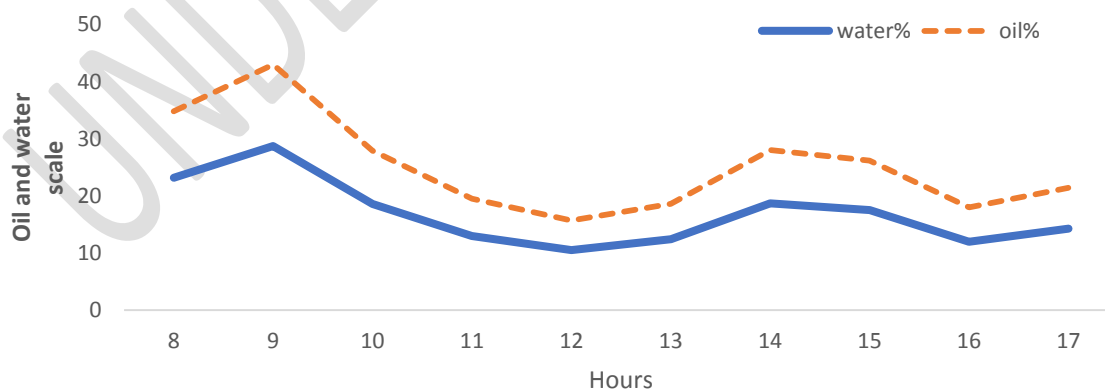


Fig 2: plot of oil and moisture content for day1(Early Sunrise Day)

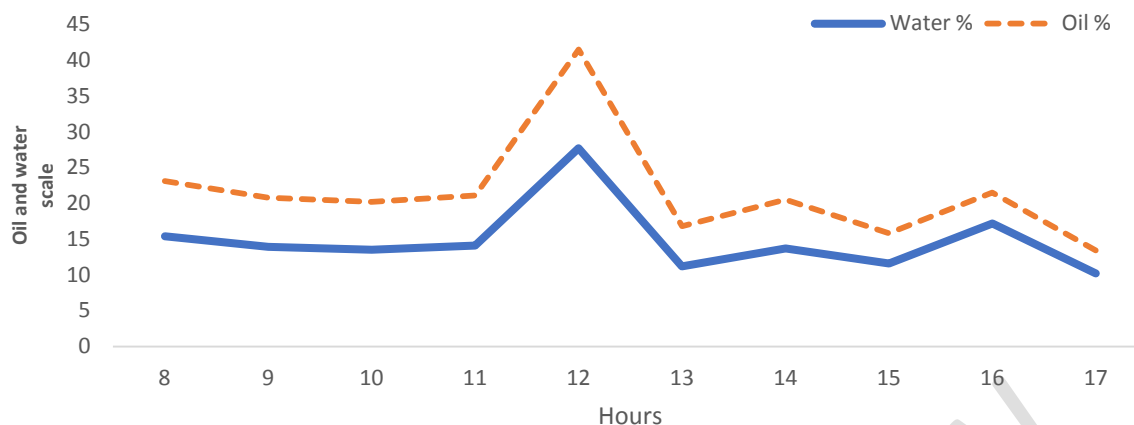


Fig 3: plot of and moisture content for day 2 (Normal bright day)

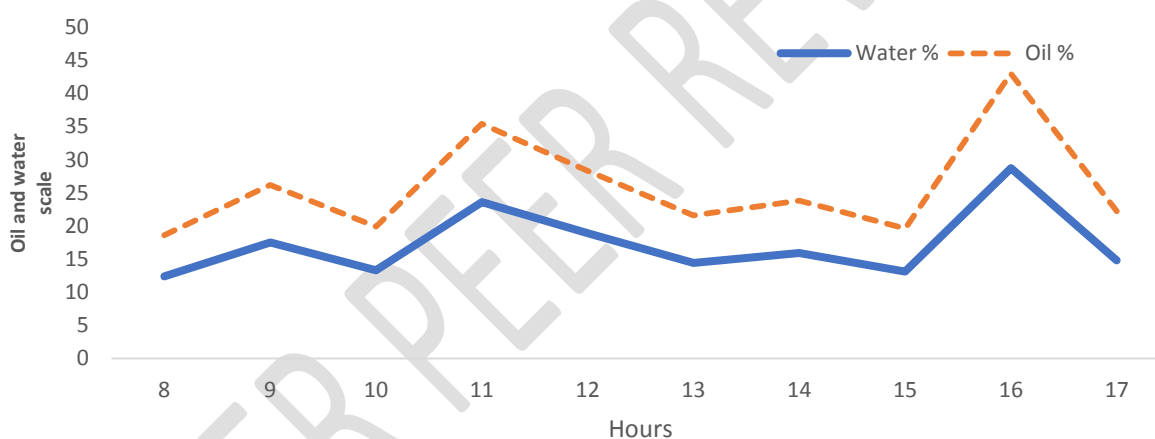


Fig 4: plot of oil and moisture content for day 3 (Raining day)

The distributions of oil and moisture content in the neem leaves depends on the Evapo-transpiration phenomenon associated with the heating effects of the sun rays and the osmotic pulling of the nutrients between the morphology of the plants transport system. This is evidenced by the peaks recorded in the repeated days with similar weather description. Therefore, the time of the day and the prevailing weather condition is very important in obtaining contents and quantity of herbal resources.

3.2 ANALYSIS OF EXTRACT

The group phytochemicals detected in the neem leaves as well as the amounts of each based on the qualitative and quantitative analysis of extract on the basis 100g macerated neem leaves is presented in Table 1:

Table 1: Quantitative Analysis of Neem contents

	Sapoin mg/ 100g	Tannin mg/100g	Flavonoid mg/100g	Phenol mg/100g	Alkaloids mg/ 100g	Reducing sugar mg/100g	Terpenoid mg/100g	Cardiac glycoside mg/100g	Steroid mg/ 100g
Sample1	34.56	31.57	31.97	43.40	29.38	33.93	14.30	39.54	15.73
Sample2	35.22	31.86	31.70	43.79	28.14	32.99	14.87	39.13	16.64
Average	34.89	31.715	31.835	43.59	28.76	33.46	14.585	39.335	16.185

3.3 Required Dosage Recommendation.

The recommended dosage of phytochemicals for human consumption was evaluated according to Toney-Butler et al (2021) as shown in equation 8:

$$\text{Dosage} = \frac{D \times Q}{H} \quad (8)$$

Where; D – prescribed intake

H – available amount

Q – quantity(1ml)

The evaluated quantities of the respective phytochemicals and flavonoids are presented in Table for references and comparison with the dosage indiscriminately consumed by herbal product users especially of the neem origin

Table 2: Required oral dosage

Phytochemicals	Average quantity mg/g	D (prescribed intake) mg/g	Dosage (ml)
Saponin	34.89	50mg/g/day(Diwan., et al 2000)	1.433
Tannin	31.715	45mg/g/day(Samanta., et al 2000)	1.418
Flavonoid	31.835	443mg/g/day(Tresserra., et al 2013)	13.91
Phenol	43.59	100mg/g/day(WHO 1994)	2.29
Terpernoid	14.585	120mg/g/day(Jessica., et al 2000)	8.23
Cardiac glycosides	39.335	0.125mg/d/day(John., et al 2000)	0.003177
steroid	16.185	10mg/g/day(Conn 2001)	0.62

4.0 CONCLUSION AND RECOMMENDATIONS

The oil and moisture content in which the phytochemicals, active ingredients and transport systems are contained was seen to follow the same pattern though in different quantity, availability of phytochemicals in oil and moisture is weather dependent, oral dosage required for human consumption has been compared to the orthodox prescription. The disparity calls for cautions as daily consumption of herbal medicine is increasing as herbal and advocates of herbal medicines and practitioner are increasing. The correct utilization of these products in the right dosages should

therefore be regulated as overdose and incorrect or incompatible combinations might have serious consequences on the short or later in the future of consumers.

Medicinal plants in general, and neem in particular, are a gift from nature, and their use should be encouraged, particularly in the production of pharmaceuticals, which provides an incentive to practice. Several contemporary medications have been discovered to have traditional origins; hence, medicinal plants have been recognized. Alternative medicine therefore should be a symbiotic relationship between traditional medical practice and its orthodox counterpart in preventing health complications. Dosage of local herbs should be controlled and evaluated to remove abuse and longtime effect. However, there was no significant dose obtained for the alkaloid, compared to the quantity required in man. This may result in requiring specific that herbal dosages calibrated either on wet/dry basis.

NOTE:

The study highlights the efficacy of "Herbal medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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