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Studies on the Effect of Temperature, Light and Storage on the Stability of Neem (*Azadirachta Indica* A. Juss) Seeds Oil Extract

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Abstract: *The stability of extracted azadirachtin was evaluated under conditions of exposure to Ultraviolet (U) light, Sunlight(S), Fridge (F), and Room temperature(R) for two weeks. Qualitative analysis of extracted azadirachtin was carried out on the sample azadirachtin using Fourier Transform Infra Red, High Performance Liquid Chromatography, Thin Layer Chromatography and the results were compared with the standard azadirachtin (reference sample).*

All the samples under the different conditions indicated significant similarities in spectra pattern and elucidated functional groups with the exception of samples stored in plain bottles under sunlight from day12-14 and ultra-violet light from day11-14 in which O-H_{str} peak was absent. The concentration (µg/ml) expressed in mean and standard error of mean (±S.E.M) were calculated as 35.07±2.04, 31.64± 2.03, 31.50± 1.81, 28.34±1.59, 29.07±1.53, 26.14±1.76, 31.71±1.75 and 28.61±2.02 respectively.The results obtained indicated storage in the refrigerator or at room temperature as the best method of preserving azadirachtin. However, storage in brown bottles is preferred to plain bottles as indicated by the result of the mean concentrations.

Key Words: *Stability, Neem seeds, Room temperature, Refrigerator, sunlight and Ultra-violet light,*

I. Introduction

Neem, Azadirachta indica is a tree in the mahogany family, Meliaceae. It is one of the two species in the genus Azadirachta and is native to Burma, India and Pakistan, growing in tropical and semi – tropical regions. It is called neem, mangosa tree or dogonyaro in Nigeria and is reputed to have several medicinal values (Gbile, 1986). Neem can grow in many different types of soil but thrives best on well drained deep and sandy soils (pH 6.2 – 7.0). It is a typical tropical / subtropical tree and exists at annual mean temperature between 21 – 3 2^0C. It can tolerate high to very high temperature (Morgan, 1989).

The stability of a drug product is the ability of a particular drug formulation in a specific container to retain its physical, chemical microbiological and biopharmaceutical properties within specified limits throughout its shelf – life (Olaniyi, 2000). The stability testing provide evidence on how the quality of an active substance or pharmaceutical product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light (WHO, 1990).

One of the first active ingredients isolated from neem, Azadirachtin has proved to be the tree's main agent for battling insects (Schmutterer, 2002). Azadirachtin is structurally related or similar to insect hormones called "ecdysones" which controls the process of metamorphosis as the insects pass from larva to pupa, to adult. Azadirachtin is a large and complex molecule with a number of reactive functional group, which renders the compound unstable under a variety of conditions. Azadirachtin is reported to decompose in the presence of acids, bases and water (Morgan, 1989). The most significant limitation to the successful use of Azadirachtin as a pesticides and insect repellant is the stability of the Azadirachtin in solution.

The plant kingdom is a vast store house of biologically active chemicals that may be suitable for control of insect pests. Insect are less likely to develop resistance to this botanical controls as their activity is multifaceted. Plant based insecticides are safer than synthetic insecticides. Neem oil is effective in controlling more than 200 species of insect pests reported by various researchers around the world and no other plant or synthetic substance has such a diverse action on insects. Millions of pounds of synthetic insecticides are being used on residential gardens and lawns and crops every year throughout the world (Vitemeyer, 1992). Usually they are quick in action and kill any insect on contact, including the beneficial insect. On the other hand, neem oil is non – toxic to animals and people. Only insects that are harmful for plant growth are affected by neem oil, leaving honey bees and other beneficial insect unharmed (Vitemeyer, 1992).

The objective of this study is to determine the stability of Azadirachtin under different storage conditions.

II. Materials And Methods

Methodology:

Sample collection:

Fresh neem seeds were picked at NARICT, Basawa, Zaria and dried at room temperature.

Oil extraction:

6.0Kg neem seeds was weighed and dehulled to obtain the husk and kernel. The kernel was crushed into powder and moistened with warm water. The moistened powder was then kept in a hot air oven in order to activate the moistened powder. The mass obtained was extracted in an oil press (hydraulic press) to give the neem oil and cake.

Extraction of Azadirachtin:

One liter of n-hexane was added to 500ml of neem oil in a conical flask. The precipitate was allowed to settle and the mixture of oil and solvent was decanted. The precipitate was washed several times with n-hexane to remove residual oil. It was then, vacuum filtered and the dried sample stored in an amber bottle. The decanted filtrate and the filtrate obtain from vacuum filtration was evaporated in a Vacuum Rotary Evaporator to obtained the oil and recover the solvent used (Jacobson, 1998).

Preparation of sample for Analysis:

Four hundred milligram (400mg) of Azadirachtin sample was weighed into eight separate glass Containers (four amber and four plain bottles).The Containers were divided into four different set, each set consisting of two containers of amber and plain bottle respectively.

Set 1- stored under room temperature

Set 2- stored in the refrigerator

Set 3- stored under sunlight

Set 4- Exposed to ultra-violet light (365nm)

This condition was maintained for fourteen days (2 weeks).

Preparation of standard curve:

Standard azadirachtin sample (1mg) was dissolved in 20ml of methanol to give 50 µg/ml stock solution of azadirachtin sample in methanol. Working concentrations of 10-50µg/ml were prepared by serial dilution in 5ml volumetric flask. Absorbance was measured using UV – Spectrophotometer at 212nm.

Preparation of sample for UV- spectrophotometric Analysis:

Extracted azadirachtin sample (1mg) was dissolved in 50ml methanol which was used for analysis in UV spectrophotometer at 212nm.

Preparation of TLC plates:

Silica gel powder (20g) was mixed with distilled water to prepare slurry for coating TLC plates. The coated plates were allowed to dry and activated in an oven set at 120^{O}Cfor 1 hr.

Preparation of sample for TLC Analysis:

Azadirachtin sample in methanol was withdrawn daily from each sample. The samples were then spotted on a TLC plate and the plates were developed in toluene: ethyl acetate (4: 1) (Jacobson, 1998).

Preparation of sample for Fourier transform infrared (F T I R) Analysis:

Azadirachtin sample (1mg) was mixed with nujol (CCl_4) for FTIR analysis.

Preparation of sample for HPLC Analysis:

A 15µL azadirachtin sample was filtered through 0.45 membrane filter and injected onto the HPLC plate using injector. The mobile phase was acetonitrile: water (35%:65%), a flow rate of 1ml/min and reversed phase column (ODS) with a UV detector set at 215nm.Only the change in retention time of azadirachtin samples was examined using HPLC.

III. Results

Percentage Yield of Azadirachtin.

0.823 % of azadirachtin was obtained.

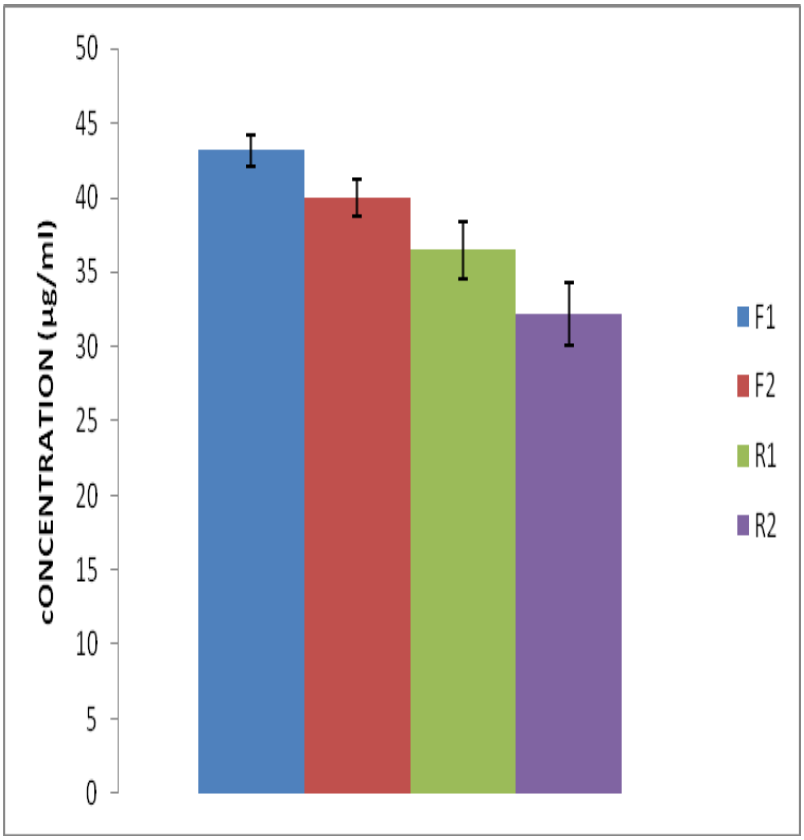

Figure1: Concentrations (µg/ml) of samples stored in refrigerator (brown bottle, F_1 and plain bottle, F_2) and at room temperature (brown bottle R_1 and plain bottle R_2) from days1-14

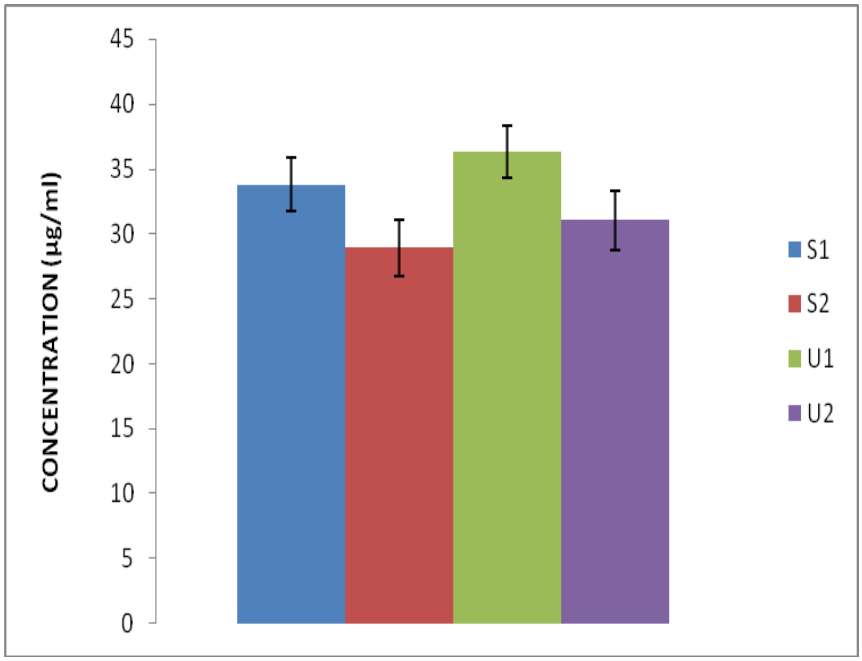

Figure 2: Concentrations (µg/ml) of samples exposed to sunlight (brown bottle, S_1 and plain bottle, S_2) and ultra-violet light (brown bottle U_1 and plain bottle U_2) from days1-14.

Table3: R_F-values of samples exposed to storage in refrigerator (brown bottle F1 and plain bottle F_2) and room temperature (brown bottle R_1 and plain bottle R_2) from days 1-14

	F_1	F_2	R_1	R_2
D	$R_{f_{F_1}}$	$R_{f_{F_2}}$	$R_{f_{R_1}}$	$R_{f_{R_2}}$
1	0.70	0.70	0.70	0.70
2	0.70	0.70	0.70	0.70
3	0.70	0.70	0.70	0.70
4	0.70	0.70	0.70	0.70
5	0.70	0.70	0.70	0.70
6	0.70	0.70	0.70	0.70
7	0.70	0.70	0.70	0.70
8	0.70	0.70	0.70	0.70
9	0.70	0.70	0.70	0.65
10	0.70	0.70	0.70	0.60
11	0.70	0.70	0.65	0.55
12	0.65	0.65	0.60	0.45
13	0.55	0.55	0.55	0.40
14	0.55	0.55	0.45	0.35

Table4: R_F values of samples exposed to sunlight brown (bottle S_1 and plain bottle S_2) and ultra-violet light (brown bottle U_1 and plain bottle U_2) from days 1-14.

D	S_1	S_2	U_1	U_2
	R_fS_1	R_fS_2	R_fU_1	R_fU_2
1	0.70	0.70	0.70	0.70
2	0.70	0.70	0.70	0.70
3	0.70	0.70	0.70	0.70
4	0.70	0.70	0.70	0.70
5	0.70	0.70	0.70	0.70
6	0.70	0.70	0.70	0.70
7	0.70	0.70	0.70	0.70
8	0.70	0.65	0.70	0.65
9	0.70	0.55	0.70	0.60
10	0.65	0.45	0.70	0.55
11	0.55	0.40	0.65	0.50
12	0.45	0.35	0.60	0.45
13	0.35	0.25	0.55	0.35
14	0.25	0.00	0.45	0.35

Fourier Transform Infra-red (FTIR) Spectra and HPLC Results:

The functional groups elucidated from the peaks of the FTIR spectra of standard and sample exposed when compared revealed Similarity in frequencies peaks. However, absence of $O\text{-}H_{str.}$ was observed in some of the samples especially samples in plain bottles in the last two days. There was no difference in the retention time of the azadirachtin standard (6min) and the samples (6min).

IV. Discussion

Percentage (%) Yield of Azadirachtin:

The percentage yield of azadirachtin was 0.823%. The value obtained corresponds with 0.2 to 0.8 percent by weight estimated by Nutan Kaushik; 2002.

UV- Spectrophotometry Analysis:

Concentrations of samples stored under room temperature, refrigerator, sunlight and ultra-violet:

There were insignificant reduction in the concentrations of azadirachtin from day1-12 and irregular concentrations were observed from day12-14, suggesting possible degradation and formation of degradative products. The concentrations of samples stored in brown bottle in the fridge (F_1) decreased from 47-37.5µg/ml, plain bottles (F_2) decreased from 47-40µg/ml, samples stored at room temperature in brown bottle (R_1) decreased from 47-36.46µg/ml, plain bottles (R_2)decreased from 47-32.18µg/ml, samples stored under sunlight in brown bottles (S_1) decreased from 47-33.79.5µg/ml, plain bottles(S_2) decreased from 47-28.93.5µg/ml and samples exposed to ultra-violet light in brown bottles (U_1) decreased from 47-36.36µg/ml and plain bottles (U_2) decreased from 47-31.04µg/ml .This was then followed by an irregular concentrations. Sample stored in brown bottle (F_1) in the refrigerator had the highest concentration. While sample stored in plain bottle under

sunlight (S_2) had the lowest concentration indicating degradation of azadirachtin as a result of exposure to sunlight. Samples exposed to ultra-violet light, also had similar results.

Student t-test was carried out on the various concentrations to compare the difference between storage in brown and plain bottles. There was no significant ($P< 0.1$) difference between storage in brown bottles and plain bottle for sample stored in the fridge. While there was significant ($P< 0.1$) difference between storage in brown bottles and plain bottle for sample stored at room temperature, under UV-light and sunlight. This suggests the need to store azadirachtin sample away from light, heat, moisture etc.

TLC Analysis:

R_F-values of samples Stored under room temperature, refrigerator, sunlight and ultra-violet light:

The results of TLC analysis of sample azadirachtin showed two distinct spot with R_f values 0.7 and 0.5 in which 0.7 for all the samples correspond to the R_f value of a single spot obtained from standard azadirachtin. R_F values for all samples and standard in brown bottles and plain bottles from day1-14 were observed. In F_1 , R_FF_1 decreased from 0.7 to 0.65 on the 14th day and in F_2, R_FF_2 decreased from 0.7 to 0.65 on the 14th day. In R_1, R_FR_1 decreased from 0.70 to 0.45 on the 14th day and in R_2, R_FR_2 decreased from 0.73 to 35 on the 14th day. In S_1 , R_FS_1 decreased from 0.70 to 0.25 on the 14th day and in S_2 ,R_FS_2 decreased from 0.70 to 0.32 on the 13th day , no spot was observed on day 14 . In U_1 R_Fu_1 decreased from 0.70 to 0.45 on the 14th day and in U_2, R_fU_2 decreased from 0.70 to 0.35 on the 14th day.

The decrease in R_F values could indicate degradation of azadirachtin and formation of derivative or new compound. The absence of spot on day 14 for sample stored in plain bottle under sunlight could also be as a result of degradation and inability of the solvent system to resolve the resulting compound.

FTIR Spectra:

The results of FTIR spectra indicated similarities between the spectrum of standard azadirachtin and sample azadirachtin showing presence of peaks which are characteristic of azadirachtin. The FTIR spectra of samples exposed to sunlight, refrigerator and room temperature showed varying peak. On day1 peaks from U_1, U_2,F_1 ,F_2, R_1, S_1 and S_2 had similar pattern and showing almost the same characteristic absorption spectrum of similar functional groups with azadirachtin standard such as presence of O-H, C-H, C=O; ketones, aldehyde, carboxylic ester, C=H, C=C; vinyl, ethyl, aromatic conjugation, methylene, aromatic groups. However, samples stored under ultra-violet light (U_2) from day 11-14, and samples stored under sunlight (S_2) from day12-14, showed absence of O-H_{str} frequency which was present in the standard and other azadirachtin samples spectra. Exposure to intense sunlight or artificial UV light could cause photochemical reactions which involves degradative reactions such as oxidation, reduction, ring rearrangement, or modification and polymerization (Greenhill etal., 1990).

The variation in spectra pattern absorption peaks suggests the degradation of azadirachtin and formation of azadirachtin derivatives.

HPLC Analysis:

Results of HPLC analysis indicated the retention time of standard azadirachtin to be 6.60 min and sample azadirachtin was 6.60min. The similarity of the retention time of standard azadirachtin with sample azadirachtin serves as an identification test and confirmed the presence of azadirachtin in the samples which were however in different concentrations. In conclusion, sample in brown bottle stored in the fridge remains the best way of storage. Also, for at least twelve days azadirachtin was found to be stable in plain and brown bottles in the fridge and at room temperature.

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