# Formulation and evaluation of the antifungal activity of Neem seed oil ointment against *Tinea capitis.*

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**ABSTRACT**

**Background**: The plant *Azadirachta indica* (Meliaceae) , a tree widely grown in Nigeria has been reported variously to have medicinal properties. The development of appropriate dosage formulation of useful therapeutic value has been a challenge.

**Objective:** The present study therefore seeks to evaluate oil from Neem seed both in its extract form and in ointment formulation against fungal organisms.

**Method**: The oil was extracted from dried seed kernel with n-Hexane, Ethanol and Chloroform respectively at seed weight to solvent ratio 1:10. The extract was evaluated as antifungal using *Tricophyton violaceum* and *Epidermophyton floccosum* as test organisms. The extract with highest antifungal activity was invariably formulated into ointment at concentrations 0%w/w, 2.5%w/w,5%w/w, 7.5%w/w & 10%w/w respectively using hydrocarbon ointment base .The ointment was *evaluated* for physical characteristics and tested against *Tricophyton violaceum* and *Epidermophyton floccosum* using Whitfield ointment as reference.

**Result**: The result showed that n-Hexane out of solvents for extraction gave the highest Neem seed oil yield (25.0%w/w) and zones of inhibition 2.1cm and 1.7cm against *Tricophyton violaceum* and *Epidermophyton floccosum* respectively. Ointment formulations gave favourable physical characteristics and had higher zones of inhibition at 7.5% w/w, 10.0% w/w against *Tricophyton violaceum* and at 5.0% w/w, 7.5% w/w, 10.0% w/w against *Epidermophyton floccosum* ($p<0.05$) than the reference standard (Whitfield's Ointment)

**Conclusion:** Neem seed oil ointment could be used in *Tinea capitis* infection

**Keywords**: *Azadirachta indica, Inhibition, Tricophyton violaceum, Epidermophyton floccosum*

# Formulation et évaluation de l'activité antifongique de la pommade d'huile de la graine de margousier contre la teigne *tinea capitis.*

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**Contexte**: La plante *Azadirachta indica* (Meliaceae), un arbre largement cultivé au Nigeria a été rapporté à maintes reprises comme ayant des propriétés médicinales. Le développement de la formulation posologique de valeur thérapeutique utile reste un défi à relever.

**Objectif:** La présente étude vise donc à évaluer l'huile provenant de la graine de margousier tant dans sa forme d'extrait que dans la formulation de la pommade contre les organismes fongiques.

**Méthode**: L'huile était extraite du noyau de la graine séchée avec du l'hexane-n, l'éthanol et le chloroforme respectivement au poids de la graine sur solvant 1:10. L'extrait était évalué comme antifongique en utilisant le *Tricophyton violaceum* et l'*Epidermophyton floccosum* comme organismes de test. L'extrait avec la plus grande activité antifongique était invariablement formulé dans une pommade à des concentrations de 0%w/w, 2,5%w/w,5%w/w, 7,5%w/w & 10%w/w respectivement en utilisant la base hydrocarbure de la pommade. La pommade a été évaluée pour les caractéristiques physiques et testée contre le *Tricophyton violaceum* et l'*Epidermophyton floccosum* à l'aide de la pommade Whitfield comme référence.

**Résultat**: Le résultat a montré que l'hexane-n parmi les solvants à extraire a donné le rendement le plus élevé d'huile de la graine de margousier (25,0%w/w) et les zones d'inhibition 2,1cm et 1,7cm contre le *Tricophyton violaceum* et l'*Epidermophyton floccosum* respectivement. Les formulations de la pommade ont donné des caractéristiques physiques favorables et des zones élevées d'inhibition à 7,5% w/w, 10,0% w/w contre le *Tricophyton violaceum* et à 5,0% w/w, 7,5% w/w, 10,0% w/w contre l'*Epidermophyton floccosum* ($p<0,05$) que la référence type (pommade de Whitfield)

**Conclusion:** La pommade de l'huile de la graine de margousier pourrait être utilisée dans l'infection de teigne *Tinea capitis*.

**Mots clés**: *Azadirachta indica*, Inhibition, *Tricophyton violaceum*, *Epidermophyton floccosum*

## INTRODUCTION

*Azadiractita indica* A Juss, synonymous with *Melia azadirachta* and *Melia indica* (A Juss), belongs to the family meliaceae. This plant is indigenous to the Indo-Pakistan subcontinent , although it is now widely distributed in many countries of the world; it is believed that Indian migrating to Africa introduced it into the continent.[1] There were so many ethno medicinal claims using different parts of Neem plant as an anthelminthic, antifeedant, antiseptic, diuretic, contraceptive, febrifuge, antimalaria, parasiticide, urticaria, antifungal, scrofula among others.[2] These folklore claims have led workers to go into extensive studies to establish these claims. Some of the findings as reported in a literature review by Aremu, Femi-Oyewo & Adeyemi [3] cited , are the use of Neem seed oil as anticandidal activity, antipyretic effect, antifungal effect , repellent effect against *Anopheles gambiae* mosquito.[4-7] One active component, gedunin gave significant control as effective as quinine against malaria.[8,9] Alcoholic extracts of bark and leaves of Neem have been used against chloroquine resistant strains of plasmodium parasite.[10,11] Neem plant as do all other plants, contain several thousands of chemicals. The most important of it are the terpenoids. More than a hundred terpenoids are known from different parts of the neem plant of its biological constituents, the most active and well-studied compound is azadirachtin. Others are nimbin, salanin, epoxyazadiradione, deacetyl salanin among others.[12] However, in most traditional preparations of Neem as pesticide or medicine, a mixture of Neem chemicals is present and provides the active principles.[12] Several different kinds of Azadirachtin (A to K) have been isolated , the most abundant of which is azadirachtin A.[12] The Neem terpenoids are present in all parts of the plant , in the living tissues. Recently, the site of synthesis and accumulation of the Neem chemicals has been identified as secretary cells. Secretary cells are most abundant with seed kernels. The secretary cells can be seen with iodine solution.[13]

*Tinea captis* has been a serious public health concern because of its easy transmissibility. It is usually caused by the species of *Microsporum, Tricophyton* and *Epidermophyton* that invade the hair shaft, endemic in many countries afflicting primarily pre-pubertal children between 6 and 10 years. It is more common in males than females. [14,15] The clinical presentation is typically a single or multiple patches of hair loss sometimes with a 'black dot' pattern (often with broken-off hairs) that may be accompanied by inflammation, scaling, pustules and itching. The disease is infectious and can be transmitted by humans, animals or objects that harbor the fungus. Carrier state also exists where the fungus is present on the scalp but there are no clinical signs or symptoms. Tricophyton violaceum and Trichophyton soudanense are common causes of Tinea capitis in parts of Africa and West Asia.[16-18] An ointment is a homogenous viscous semi-solid preparation most commonly a greasy thick oil with a high viscosity that is intended for the application of active ingredients to the skin or mucous membranes. They are used as emollients for the application of active ingredients to the skin for protective, therapeutic or prophylactic purpose and where a degree of occlusion is desired.[19] There is the need to channel various discoveries of traditional use of Neem parts into a delivery design that will be convenient for administration through oral or dermal route. There have been reported cases of bacteria and fungi resistance to some synthetic formulations some of which are even causing adverse skin reactions.[20]

In the present study, Neem seed oil is being designed in a dermatological ointment formulation, evaluate its physico-chemical characteristics and then screen for antifungal activity against Tinea capitis.

## METHODS

**Collection of neem plant seeds:** Ripe seeds of Neem plant were collected from different Neem trees in different locations using systematic sampling approach in Ibadan Polytechnic, Oyo State,Nigeria between the hours of 10-11a.m. The plant species, from which the seeds were collected, was authenticated at Forest Research Institute of Nigeria (FRIN) with FHL No 107818 . The seeds were sun dried until the moisture content was reduced to about 10%w/ using moisture analyzer. The seed kernels were later separated from the seed coat and stored in air tight containers. The dried seed kernels were comminuted using blender model Mx-738 (Nakai, Japan) and stored in air tight containers. Three solvents (n – hexane, ethanol and chloroform) were used for extraction at seed weight: Solvent ratio 1:10. The pulverized seeds were macerated in the solvents for 8 days at room temperature and filtered through a whatman filter paper (No.1) to remove the coarse seed materials into pre-weighed sterile containers. The solvents were evaporated using rotary evaporator. The weight of extract residue was then determined and kept at room temperature.

**Characterization of the extracted oil**

The melting point, boiling point, specific gravity, refractive index, pH, acid value, free fatty acid value were determined using standard methods . The determinations were carried out in duplicates.

**Assessment of antifungal effect of neem seed oil extract**

Neem seed oil extract was tested against clinical isolates of Tricophyton violaceum and Epidermophyton floccosum using agar pour plate method. The clinical isolates obtained were culture on Saboraud dextrose agar and incubated at 25°C for 10 days. Thereafter a loopful of the culture was transferred into 40% sucrose solution and then mixed thoroughly. One millilitre of each organism dispersed was added to 19mls of sterilized agar solution, mixed thoroughly and later poured into petri dishes for setting. The set plates were dried in a hot air. After drying, 2 cups were bored using a sterile cork borer (5mm in diameter) on each plate ensuring that the cups were far apart as much as possible and not too close to the edge of the plate. Using sterile Pasteur pipettes, 2.5g amount of extract from the different solvents and castor oil (control) were introduced into the labeled cups and the plates were incubated at 25°C for 48hrs to determine the extract with highest activity for subsequent formulation. At the end of incubation period, the plates were observed for clear zones of inhibition. This experiment was repeated once.

**Preparation of the neem seed oil ointment**

Neem seed oil extract from n-hexane solvent that gave highest mean zones of inhibition of 2.1cm and 1.7cm for Tricophyton violaceum and Epidermophyton floccosum respectively was incorporated into hydrocarbon type ointment base to get the desired test concentrations of 0%w/w, 2.5% w/w,5.0% w/w, 7.5% w/w and 10% w/w using fusion method.

**Table 1. Neem seed oil ointment formulations.**

| Ingredients | NSOI (g) | NSO2 (g) | NSO3 (g) | NS04 (g) | NS05 (g) |
|---|---|---|---|---|---|
| Ointment base | 25.0 | 24.40 | 23.80 | 23.10 | 22.5 |
| Neem seed oil | 0.0%w/w | 2.5%w/w | 5.0%w/w | 7.5%w/w | 10.0%w/w |

Keys:

NSO1 = Neem seed oil ointment (0.0%w/w) NSO4 = Neem seed oil ointment (7.5%w/w)
NSO2 = Neem seed oil ointment (2.5%w/w) NSO5 = Neem seed oil ointment (10.0%w/w)
NSO3 = Neem seed oil ointment (5.0%w/w)

**Evaluation of drug release**

Exactly 0.25ml of melted ointment was measured into a 25ml volumetric flask and made up to 25ml with phosphate buffer and then mixed thoroughly. Sterilized nutrient agar was poured into plates and allowed to solidify, the surface of each plate was flooded with ferric chloride solution 50% w/v and the excess solution was drained off. Three cups were bored in this plates using cork borer. 0.5ml of different concentrations (0% w/w, 2.5% w/w, 5% w/w, 7.5% w/w and 10% w/w) of the ointment samples were placed in the holes. This was done in duplicate. The plates were then placed on a laboratory bench for 30mins for diffusion to take place before being incubated at 25°C and 37°C. The zones of colour changes were measured for each sample at time intervals of 1, 2, 3, 12 and 24 hours respectively.

**Assessment of antifungal effect of neem seed oil ointment**

Neem seed oil ointment was evaluated against clinical isolates of *Tricophyton violaceum* and *Epidermophyton floccosum* in duplicate using pour plate method as in the case of assessment of oil extract. The oil extract was replaced with Neem seed oil ointment formulations.

**RESULTS**

**Neem seed yield**

The Neem seed kernel yields an acrid bitter greenish yellow to brown fixed oil. The calculated yield is between (19-25%$^{w}/_{w}$). The results and characterization of the extracted oil using 3 solvents is presented in Table 2.

**Table 2: Characteristics of Neem seed oil extract.**

| Samples | Mean Boiling point ($^0$C) | Mean Melting point | Mean Refractive index | Mean Ph | Meaan Specific gravity | Mean acid value (ml/g) | Mean Free Free Fatty Acid Value (ml/g) | Colour / Odour |
|---|---|---|---|---|---|---|---|---|
| HOE | 250±1.0 | 28±1.0 | 1.368±0.002 | 9.4±0.1 | 0.904±0.001 | 4.07±0.110 | 239.70±1.15 | Brown/Garlic Like |
| EOE | 252±1.5 | 27±0.9 | 1.366±0.001 | 9.3±0.2 | 0.922±0.001 | 4.77±0.21 | 282.00±2.12 | Brown/Garlic Like |
| COE | 249±1.0 | 28±0.8 | 1.366±0.001 | 9.0±0.1 | 0.916±0.002 | 5.05±0.35 | 267.9±1.25 | Brown/Garlic Like |
| CSO (control) | 50±0.5 | 12±0.3 | 1.477±0.001 | 8.9±0.1 | 0.953±0.002 | 3.85±3.85 | 221.25±1.36 | Colourless/Slight odour |

Keys:
HOE = n-Hexane oil extract
EOE = Ethanolic oil extract
COE = Chloroform oil extract
CSO = Castor oil

The results of drug release from ointment base determined at 25$^o$C and 37$^o$C by measurement of diameter of zone of colour change is presented in Table 3 and Plates 3 and 4.

**Table 3: Mean *in-vitro* drug release of Neem seed oil ointment.**

| **Sample** | **Time(hours) at 25ºC** | | | | | **Time(hours) at 37ºC** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1 (cm)** | **2 (cm)** | **3 (cm)** | **12 (cm)** | **24 (cm)** | **1 (cm)** | **2 (cm** | **3 (cm)** | **12 (cm)** | **24 (cm)** |
| NSO1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| NSO2 | 0.30 | 0.50 | 0.80 | 1.10 | 1.10 | 0.30 | 0.90 | 1.20 | 1.35 | 1.35 |
| NSO3 | 0.30 | 0.70 | 1.00 | 1.60 | 1.60 | 0.30 | 0.90 | 1.50 | 2.00 | 2.00 |
| NSO4 | 0.40 | 1.00 | 2.00 | 2.70 | 2.70 | 0.40 | 1.00 | 2.00 | 3.0 | 3.00 |
| NSO5 | 0.50 | 1.50 | 2.50 | 3.25 | 3.25 | 0.40 | 1.30 | 2.50 | 3.8 | 3.80 |

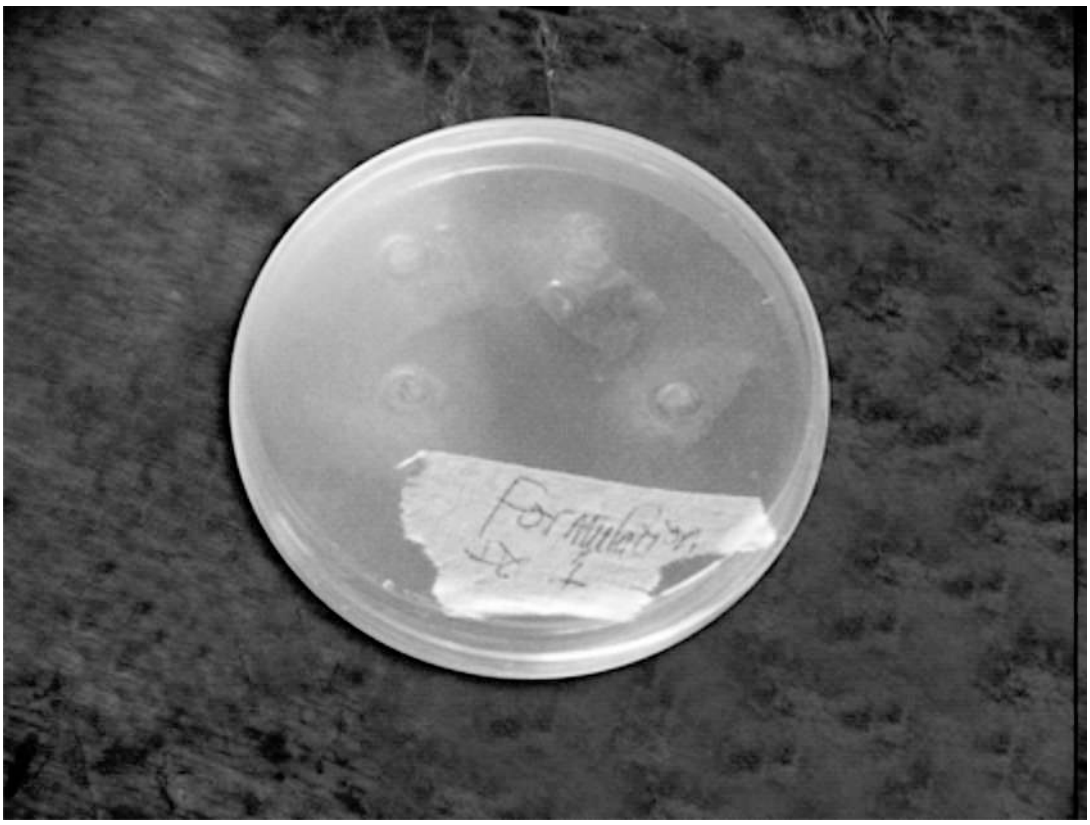

**Plate 3:*In-vitro* Drug Release of Neem Seed Oil Ointment at 25$^{0}$C**

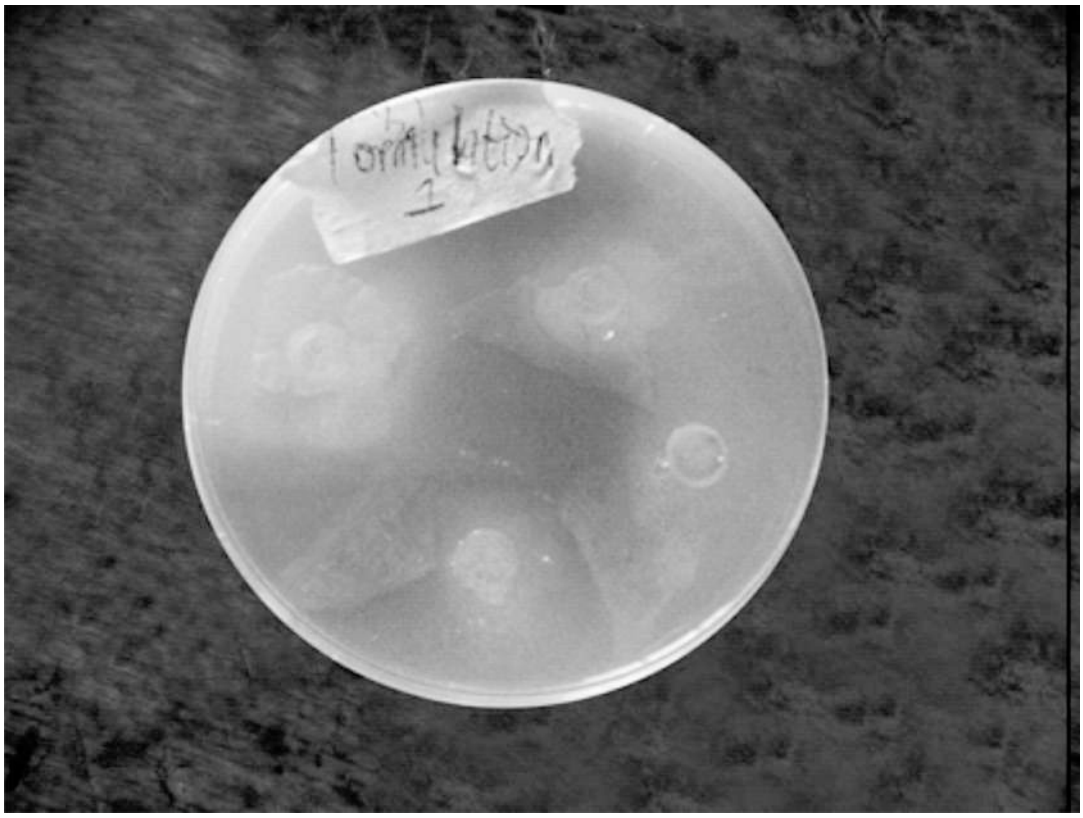

**Plate 4: *In-vitro* Drug Release of Neem Seed Oil Ointment at 37$^{0}$C**

The outcome of antifungal assessment of Neem seed oil extracts and ointment formulations is presented in Table 4.

**Table 4. Mean zones of inhibition of neem seed oil and ointment formulation.**

| Samples | Organisms | |
|---|---|---|
| | *Tricophyton violaceum* | *Epidermophyton floccosum* |
| | Mean (cm) | Mean (cm) |
| HOE | 2.10±0.01 | 1.70±0.02 |
| EOE | 1.55±0.01 | 1.35±0.12 |
| COE | 1.20±0.02 | 1.10±0.01 |
| NSO1 | 0.30±0.01 | 0.20±0.01 |
| NSO2 | 0.50±0.01 | 0.40±0.02 |
| NSO3 | 0.70±0.01 | 0.70±0.01 |
| NSO4 | 1.00±0.02 | 0.90±0.01 |
| NSO5 | 1.50±0.03 | 1.00±0.02 |
| Whitfield ointment(ref) | 0.8±0.01 | 0.6±0.02 |

Keys:
HOE = n-Hexane oil extract
EOE = Ethanolic oil extract
COE = Chloroform oil extract
NSO1 = Neem seed oil ointment (0.0%w/w)
NSO2 = Neem seed oil ointment (2.5%w/w)
NSO3 = Neem seed oil ointment (5.0%w/w)
NSO4 = Neem seed oil ointment (7.5%w/w)
NSO5 = Neem seed oil ointment (10.0%w/w)

**DISCUSSIONS**

Neem seed kernel yields an acid bitter greenish yellow to brown fixed oil with garlic odour. The calculated yield is 19 – 25% w/w. The variation in yield is probably a reflection of both the solvents of extraction and different locations from where Neem seeds were collected in Ibadan, Nigeria. Ibadan is situated on a highland plateau ranging from500ft to 1200ft (152.5 to 366 metres) above sea level. There are two seasons, the rainy and the dry. The rainy season characterized by the south-westerly monsoon rains, usually starts in late March/ early April and ends in September. The heaviest rainfall is recorded in the months of July and August, when rivers are flooded. The rainy season has an annual rainfall of between 1143mm and 1524mm. The dry season, influenced by the north easterly dry trade winds from sclera, starts around middle September and continues until March. Near the end of December, Ibadan in Nigeria normally experiences the harmattan which lasts for about a month. This is the period with the greatest daily temperature range, when the mean maximum daily temperature is between 32°C and 35°C and the mean minimum night-time temperature is between 20°C and 22°C. The soil of Ibadan is rich in humus where forest covers remains, with soil types including clay, laterite, loam and sandy soil. The ecological factors above are able to influence the yield of Neem seed oil.

Mordue & Nisbet [21] cited, reported that mature Neem tree is able to produce 2kg seed per tree per year. This could reach maximum yield of 50kg seed per tree per year ten years after.[22-24] Considering the yield of 19-25% w/w which translates to 380-500g oil per Neem tree per year that has potential of reaching 9.5 – 12.5kg. Neem oil per tree per year and in view of the fact of ease of cultivation and ability of Neem tree to withstand drought conditions, this natural yield is able to sustain a continuous pharmaceutical production

The results of boiling point, melting point, refractive index, pH, specific gravity, mean acid value, mean free fatty acid value of Neem seed oil is as presented in Table 2.The boiling point of a liquid varies depending upon the surrounding environmental pressure. Melting is a physical process that results in the phase change of a substance from a solid to liquid. These physical properties especially the boiling point can be raised or lowered by the presence of impurities which can be present in the seeds or solvents used. The boiling and melting points of Neem oil determined is 249°C – 252°C and 27°C – 28°C respectively. This compares with the work of Kovo . [25] The oil extract from three solvents of extraction had refractive index ranging between 1.366 –

1.368. The refractive index is the ratio of velocity of light in a vacuum to its velocity in the substance. The measurement of refractive index is employed for pharmacopoeia purposes mainly to establish the identity of liquid substances and to detect impurities in substances particularly in fixed and volatile oils. The refractive index of oil is dependent on their molecular weight, fatty acid chain length and the degree of unsaturation. The values for specific gravity, mean acid value, mean free fatty acid value for the oil extract are 0.904 – 0.922, 4.07ml/g – 5.05ml/g, and 239.7ml/g – 282.0ml/g respectively. The essence of these determinations is one of the ways to establish the purity of the neem seed oil extract. Presence of impurities or any form of adulteration would markedly alter the values obtained for these characteristics.

The result of drug release from the different concentrations of ointment prepared using oil extract from n-hexane solvent is as shown in Table 3 and Plates 3 and 4 respectively. The zones of colour change increased in diameter with increase in concentration and time for all concentrations studied. The amount released and the rate of release of a drug suspended in a vehicle such as an ointment may be related to time and to variables of the system.[26] The rate of release of Neem seed oil is greatly influenced by diffusion co-efficient, concentration and solubility of the oil in the ointment base. Employing the following, equation ,

$$\frac{} \cong \left(\frac{AD_vC_s}{2t}\right)^{\frac{1}{2}} \text{ ..... eqn (1)}$$

Where $d_m/d_t$ is rate of release, A is total amount of ointment base, $D_v$ is the diffusion co-efficient in the base, $C_s$ is the solubility of the drug in the vehicle and t is time. It may be possible to predict *in-vitro* availability of medicament from vehicle base. Temperature also had effect on the rate of release of Neem oil at various concentrations, higher at 37°C than 25°C as can be seen in Table 3 and Plates 3 ,4. This is possibly so because temperature is able to alter diffusive tendency of drugs in ointment base at elevated temperature due to increase in fluidity of the base

The antifungal effect of the Neem seed oil extract from the three solvents used for extraction against *Tricophyton violaceum* and *Epidermophyton floccosum* is of the order n-Hexane > Ethanol > Chloroform with the mean zones of inhibition, 2.1cm, 1.55cm, 1.2cm for *Tricophyton violaceum* and 1.7cm, 1.35cm, 1.1cm for *Epidermophyton floccosum* respectively as can be seen in Table 4. Nimbidin and Gedunin according to Murthy& Sirsi [27] have been shown to possess higher antifungal effect than the rest of the bioactive compounds in the oil. It follows therefore that n-hexane of the three solvents probably had highest concentration of these substances which may be due to structural affinity for the solvent. This may have accounted for the observed antifungal trend. However further work will be necessary to elucidate on the contents and concentrations of bioactive compounds present in the oil extract using different solvents. The outcome of antifungal evaluation of Neem seed oil ointment is also presented in Table 4 for comparism. It was observed that the zones of inhibition against *Tricophyton violaceum* and *Epidermophyton floccosum* is of increasing order as the concentration of Neem seed oil was increasesing from 0% w/w to 10-% w/w. This is expected since the concentration of bioactive compounds present was increasing. As can be seen in Table 4, Neem seed oil ointment had higher zones of inhibition at 7.5% w/w, 10.0% w/w against *Tricophyton violaceum* and at 5.0% w/w, 7.5% w/w, 10.0% w/w against *Epidermophyton floccosum* ($p<0.05$) than the reference standard (Whitfield's Ointment) used in this study. This is an indication of better efficacy of the naturally occurring bioactive formulation at the stated concentrations than the synthetic one in the reference sample used. From Table 4, it could also be seen that zones of inhibition of Neem seed oil ointment appear lower than that of Neem seed oil . This is because Neem seed oil would have been distributed into different structural network of the base which would require time for its diffusion out of the base before it could elicit activity.

**CONCLUSION**

The natural yield of Neem seed oil (19-25%w/w) in this study is adequate to support continuous production of Neem seed oil ointment. The seeds abound every zone of this country to support commercial activity. The ointment formulated satisfied standard requirements without any difficulty in drug release from the base. Successful formulation of this ointment will lead to other delivery designs of Neem seed oil that will address other therapeutic conditions **.** Neem seed oil ointment formulations had higher zones of inhibition at 7.5% w/w, 10.0% w/w against *Tricophyton violaceum* and at 5.0% w/w, 7.5% w/w, 10.0% w/w against *Epidermophyton floccosum* ($p<0.05$) than the reference standard (Whitfield's Ointment). Neem seed oil ointment may be recommended as useful alternative in the treatment of *Tinea capitis*

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