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# Acetyl and Butyrylcholinesterase Inhibiting Constituent from *Morinda lucida* Benth (Rubiaceae)

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***Authors' contributions***

*This work was carried out through collaboration between all authors. Author SAA designed the study and wrote the protocol. Author JMA contributed to the protocol and provided general supervision of the study. Author EO managed the experimental process and provided technical assistance. Author TOE managed the literature searches, performed the experiments, and wrote the first draft of this paper. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This study identified the cholinesterase inhibitory principle in *Morinda lucida,* one of the plants used in Nigerian ethnomedicine as memory enhancer.
**Study Design:** *In vitro* anti cholinesterase assay and spectroscopic analysis of isolated compound.
**Place and Duration of Study:** Department of Pharmacognosy, Obafemi Awolowo University, Nigeria between 2010 and 2012.
**Methodology:** Activity directed phytochemical analysis using Thin Layer Chromatography (TLC) bioautographic assay and repeated Vacuum Liquid Chromatography followed by Preparative Thin Layer Chromatography (PTLC) on silica gel was used to isolate one compound from the most active fraction of the plant.
**Results:** Spectroscopic analysis ($H^1$ and $^{13}C$ NMR) and comparison with literature data identified

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this compound as phytol (3, 7, 11, 15-tetra methyl- 2-hexadecen-1-ol), with an $IC_{50}$ of 12.93 µg/ml acetyl cholinesterase (AChE) and 24.90 µg/mlbutyryl cholinesterase (BuChE).
**Conclusion:** Isolation of compound with cholinesterase inhibitory activity has to some extent validated the ethnomedical use of the plant as memory enhancer and has provided new information on the chemistry of the plant.

*Keywords: Morinda lucida; acetyl cholinesterase; butyryl cholinesterase; phytol.*

## 1. INTRODUCTION

The use of plant, plant extracts or plant derived pure chemicals to treat disease is a practice which has stood the test of time [1] and today, there are many important drugs in clinical use that are from plants or from starting molecules of plant origin. Several authors have mentioned the importance and potential of medicinal plants as sources of new therapeutic (biological and medicinal) agents [2-5].

Some of these agents are derived from their respective plants based on ethno medical correlations e.g. acetyl digoxin, a cardiotonic from *Digitalis lanata* and monocrotaline, an antitumor agent from *Crotolaria sessilifora* (L). However, plant derived drugs developed not on the basis of ethno medical information also exist e.g. Papaverine from *Papavasomniferum* and thymol from *Thymus vulgaris* [6].

Inhibition of acetyl and butyryl cholinesterase is a major approach to the management of dementia considering the cholinergic hypothesis which correlated the degree of cholinergic deficit with severity of dementia [7,8]. Therefore, inhibitors of acetyl cholinesterase (AChE), which enhance cholinergic transmission by reducing the enzymatic degradation of acetylcholine, are approved drugs for the management of Alzheimer's disease (AD) [9-11].

Cholinesterase inhibitors are known to occur in plants used traditionally for failing memory and other cognitive declines associated with age [12]. Various researchers have shown that some of these plants possess AChE inhibitory property that may be relevant in the treatment of neurodegenerative disorders like AD. For instance, *Bacopa monniera* and *Ginkgo biloba* are well known as cognitive enhancers in Indian and Chinese traditional medical system respectively [13]. Also reported is *Acorus calamus* from Korean traditional medicine [14], *Stephania suberosa* and *Tabe mae montana divaricate* from Thai traditional medicine [15,16].

Phytochemical studies on a number of these plants have led to the isolation of active compounds with anti-cholinesterase and memory enhancing properties e.g. cynatroside from *Cyanchum atratum* [17], zeatin from *Fiatouavillosa* [18], hamayne from *Crinum* species [19].

In a study on cholinesterase inhibitory activities of medicinal plants used traditionally as memory enhancer in Nigeria, *Morinda lucida* was found to be active [20]. One of the active constituents is therefore isolated and identified in this study as phytol.

## 2. MATERIALS AND METHODS

### 2.1 Extraction and Precipitation

Fresh leaves (1 kg) of *Morinda lucida* Benth (collected from the medicinal farm of Obafemi Awolowo University, Nigeria and authenticated at the Department of botany, with herbarium number IFE 5672) was extracted using 100% ethyl acetate. The lipid constituents of the ethyl acetate extract was precipitated out using methanol. Both the precipitate and supernatant were tested for cholinesterase inhibitory activity.

### 2.2 Acetyl Cholinesterase Assay Procedures

The acetyl cholinesterase (AChE) assay was performed according to the colorimetric method of Ellman [21] and the TLC bio autographic method of Rhee [22].

The reaction assay mixture consisted of 2000mL 100mM phosphate buffer pH8.0, 100 mL of test sample stock solution in methanol (at a final concentration of 42.5 µg/ml), 100 mL of enzyme acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) solution at a final concentration of 0.003 µ/ml and 0.001 µ/m lrespectively. 100 µL of DTNB (0.3 mM) prepared in 100 M phosphate buffer pH 7.0 containing 120 mM sodium bicarbonate. The reaction mixture

was vortexed and then pre-incubated in a water bath at 37°C for 30 minutes. The reaction was initiated by the addition of 100 µL of acetyl thiocholine iodide (ATChI) or butyrylthiocholine chloride (BTChCl) at a final concentration of 0.5mM. As a negative control, the inhibitor solution was replaced with methanol. The change in absorbance at λmax 412 was then measured for a period of 5 minutes at ambient temperature. All assays were carried out in triplicate. Eserin ((-) physiostigmine) was used as positive control. The percentage inhibition was calculated as follows:

$$\% = \frac{a-b}{a} \times 100$$

Where a = ΔA/min of control
b = ΔA/min of test sample
ΔA = change in absorbance

The crude methanolic extract, the various fractions, the ethyl acetate extract, the precipitate and the supernatants were subjected to this test.

The TLC bioatographic assay was carried out by spotting the various samples on precoated (G60 PF 254) TLC aluminum plate followed by development in appropriate solvent system. The developed plates were air dried and first sprayed with $2.55 \times 10^{-3}$ units/ml of the acetylcholinesterase enzyme until saturated, incubated at 37°C for at least 20 minutes and then sprayed with 0.5 mM of the substrate (ATChI or BTChCl) and then DTNB [22].

## 2.3 Isolation of Phytol

The most active supernatant (36.66 g) was subjected to repeated Vacuum Liquid Chromatography (VLC) on silica gel using N-hexane, dichloromethane and methanol as solvent system. A total of 113 sub- fractions were collected and bulked into seven (7) based on their chromatographic pattern. The seven bulked fractions were tested for AChE inhibitory activity using TLC bio- autographic method. The result is as presented in Fig. 2.

The fraction showing highest activity ($M_1$) was further purified by VLC. 80 sub- fractions bulked into 7 were obtained. The active sub- fraction ($M1_b$) (Fig. 4) was subjected to PTLC and two compounds ML-1 and ML-2 were isolated.

## 2.4 Analysis of Isolated Compound

Compound ML-2 was subjected to spectroscopic analysis ($^1$H NMR, $^{13}$C NMR). The TLC analysis in different solvent and solubility test in water were also determined.

## 2.5 $IC_{50}$ of Phytol

The concentration of compound that is required for 50% enzyme inhibition ($IC_{50}$) was calculated from a linear estimation of the enzyme inhibition dose - response curve [23].

## 2.6 Statistical Analysis

Data were expressed a mean±SD. Statistical analysis was performed by student's T-test using sigma plot$^{(R)}$ software (Jandel scientific, Germany)

## 3. RESULTS AND DISCUSSIONS

Activity - directed fractionation using the TLC autobiography of the *M. lucida* supernatant was subjected to a combination of repetitive Vacuum Liquid Chromatography (VLC) and Preparative Thin Layer Chromatography (PTLC).

A bulked sample from the VLC of *M. lucida* supernatant is shown in Fig.1. Positive detection using vanillin/$H_2SO_4$ was indicated by a number of colours [24]. The plate showed different colours with vanillin/$H_2SO_4$ indicating the presence of many compounds which may include terpenes, steroids or essential oils.

Fig. 2 shows the results of the AChE assay for sub fractions from the VLC of *Morinda lucida* supernatant. White spot on a yellow background in the assay for cholinesterase inhibitors using TLC bioautographic indicates a positive result. From the plate below, activity can be seen on spots M1, M2, M5, and M6 with spot M1 being the most active.

Sub fraction M1 was further purified using VLC. The various sub fractions obtained from this purification process (Fig. 3) were also tested for AChE inhibitory activity.

It was observed that all the sub fractions of M1 (M1a-g) showed activity (Fig. 4). M1b was therefore subjected to PTLC to isolate compound ML-2.

The isolated compound was subjected to spectroscopic analysis. The spectra data is as presented in Table 1 below.

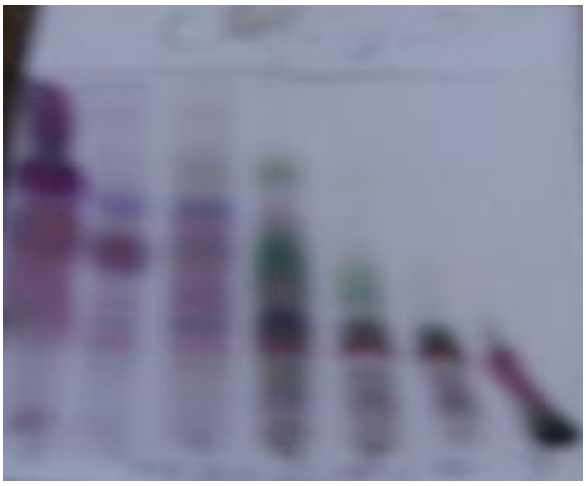


**Fig. 1. Bulked samples from the VLC of *Morinda lucida* supernatant (Vanilin/$H_2SO_4$)**
*Solvent system: Chloroform 100%; Spray: Vanillin/$H_2SO_4$*

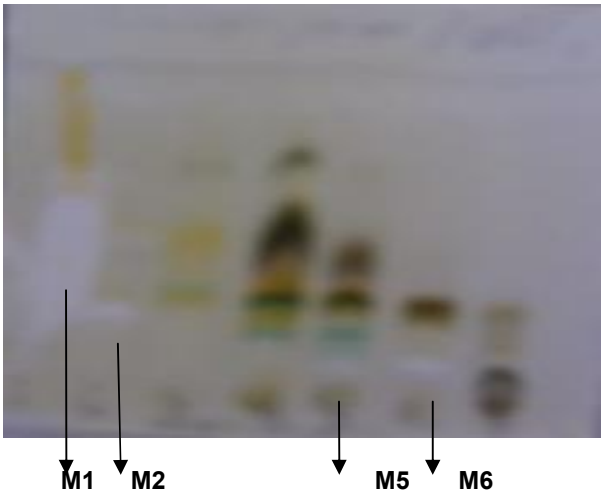



**Fig. 2. AChEAssay of Bulked fractions from the VLC of *Morinda lucida* supernatant**
*Solvent system: Chloroform 100%; Spray: Enzyme, ATCHI and DTNB*

**Fig. 3. Bulked samples from the VLC of Subfraction M1 (Vanilin/$H_2SO_4$)**
*Solvent system: Chloroform 100%; Spray: Vanillin/$H_2SO_4$*

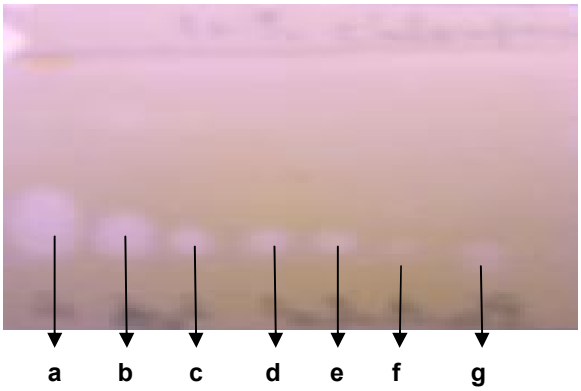



**Fig. 4. AchEAssay of Bulked samples from the VLC of Subfraction M1**
*Solvent system: Chloroform 100%; Spray: Enzyme, ATCHI and DTNB*

**Table 1. $^{1}H$ and $^{13}C$ spectra data for compound ML-2**

| | $^{1}H$ | $^{13}C$ | Integral | [25] |
|---|---|---|---|---|
| C-1 | 4.13(d) | 59.65 | $CH_2$ | 59.39 |
| C-2 | 5.39(t) | 123.30 | CH | 124.09 |
| C-3 | | 140.55 | C | 140.23 |
| C-4 | 1.98(m) | 40.10 | $CH_2$ | 39.85 |
| C-5 | 1.44(m) | 25.36 | $CH_2$ | 25.12 |
| C-6 | 1.30(m) | 36.89 | $CH_2$ | 36.65 |
| C-7 | 1.44(m) | 32.92 | CH | 32.67 |
| C-8 | 1.30(m) | 37.66 | $CH_2$ | 37.35 |
| C-9 | 1.30(m) | 24.70 | $CH_2$ | 24.45 |
| C-10 | 1.30(m) | 37.51 | $CH_2$ | 37.41 |
| C-11 | 1.44(m) | 33.01 | CH | 32.77 |
| C-12 | 1.30(m) | 37.59 | $CH_2$ | 37.28 |
| C-13 | 1.30(m) | 25.02 | $CH_2$ | 24.78 |
| C-14 | 1.14(m) | 39.59 | $CH_2$ | 39.35 |
| C-15 | 1.52(m) | 28.20 | CH | 27.95 |
| C-16 | 0.87(m) | 22.94 | $CH_3$ | 22.60 |
| C-17 | 0.87(m) | 22.85 | $CH_3$ | 22.69 |
| C-18 | 0.86(m) | 19.97 | $CH_3$ | 19.69 |
| C-19 | 0.85(m) | 19.94 | $CH_3$ | 19.72 |
| C20 | 1.65(s) | 16.41 | $CH_3$ | 16.14 |

The $^{13}C$ spectrum of ML-2 showed that there were $5CH_3$, $10CH_2$, 3CH and 1C=C. Thus, compound ML-2 appears to be a C-20 carbon compound.

The $^{1}H$ NMR spectrum had a signal at δ 5.4(t) which represents the olefinic proton assigned to the proton on C-2 in the molecule. The signal at δ 4.1(d) represents an alcohol proton and is assigned to the proton residing on C-1. A triplet signal at δ1.98 stands for the proton on C-4. The multiplet signals at δ 1.44 and δ1.35 represent the methine protons on C-7 and C-11. The third methine proton on C-15 had signal showing at δ1.52. This is due to the shielding effect of the methyl group on C-16. However, the multiplet at δ1.30 to δ1.03 is assigned to the protons on C-6, C-8, C-9, C-10, C-12 and C-13.

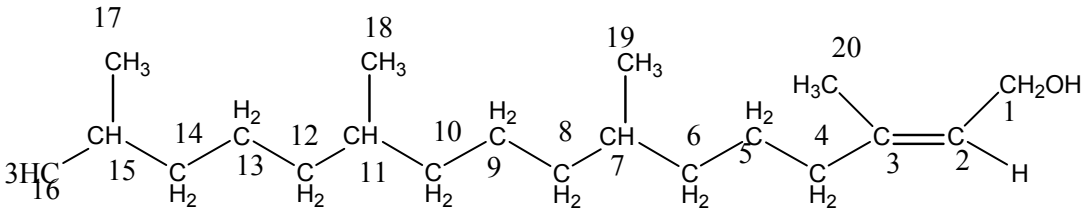
The signal at δ1.65 (s) was assigned to the methyl proton on C-20 while the signal at δ1.66 was assigned to the OH group. The signal at δ1.14 (m) was assigned to the protonon C-14 while the remaining signals at δ0.87. 0.86 and 0.85 were assigned to the methyl protons on C-17, C-18, and C-19 respectively. Analysis of the spectra showed that compound ML-2 is phytol when compared with literature values [25].

To ascertain the purity of the isolated compound, thin layer chromatography (TLC) was carried out in two different solvent systems and using two different detecting reagents (Table 2), both of which gave single spots. The solubility in water and melting point determination were also carried out. The melting point falls within the standard range for phytol thus further confirming the identity and purity.

**Table 2. TLC profile of ML-2**

| Detecting Agent | Solvent system | Colour | Rf |
|---|---|---|---|
| Vanillin/ $H_2SO_4$ | Hexane – Chloroform (50:50) | Purple | 0.56 |
| Anisaldehyde | Chloroform (100%) | Pink | 0.24 |

*Solubility in water: Insoluble; Boiling point: 202-204$^0$C; $IC_{50}$: 12.93 µg/ml (AChE), 24.90 µg/ml (BuChE)*
*ML-2= 3, 7.11.15-tetramethyl-2-hexadecen-1-ol*



*Formula: $C_{20}H_{49}O$*
*Molecular weight: 296.54*

Phytol has been previously reported in several plant species including *Laurenciatristicha* [26], red alga *Corallina pilulifera* [27], *Alternanthera philoxeroides* [28], *Isodon eriocalyx Var. laxiflora* [29], *Hypericum perforatum* [30], *Lactuca sativa* [31], *Cedrela sinensis* [32], *Ipomea pescarprae* [33], *Jolyna laminarioides* [34], and *Morinda citrifolia* [35]. Several biological activities have also been associated with the compound. These include antitubercular [36], antimycobacteria [37,38], anti-anthritis [39], and modulation of arterial thrombosis [40], It is also known to possess anti-spasmodic activity [33], and inhibits succinic semialdehyde dehydrogenase (SSADH) [31], It also acts as HIV-1 integrase inhibitory substance[41].

The compound 3,7,11,15-tetramethyl-2-hexadecen-1-ol (Phytol) is however being reported in *Morinda lucida* for the first time and linked with cholinesterase inhibitory activity for the first time with an $IC_{50}$ of 12.93 µg/ml (AChE) and 24.90 µg/ml (BuChE).

## 4. CONCLUSION

The cholinesterase inhibitory constituent of *M. lucida* has been successfully identified as phytol with good $IC_{50}$ values for both AChE and BuChE. Thus the use of *M. lucida* as memory enhancer in Nigerian ethnomedicine has been justified in this research.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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