Investigation of Chemical, Genotoxic and Haematological Properties of Secondary Metabolites from n-Hexane Extract of *Olax subscorpioidea* Oliv. (Olacaceae) Leaves

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Due to their phytochemical diversity, plants may exhibit various biological activities. They may be medicinal as well as toxic. As a result, investigating their toxicities is as important as investigating their medicinal properties. This study investigated the chemical, genotoxic and haematological properties of the *n*-hexane extract of *Olax subscorpioidea* leaves. The chemical investigation of the extract was done by gas chromatography-mass spectrometry (GC-MS) analysis. Genotoxic and haematological investigation were done using *in vivo* models in mice. Genotoxic effects were estimated by evaluating DNA fragmentation with agarose gel electrophoresis and diphenylamine (DPA) assays. Haematological indices were measured on an Auto Haematology Analyzer. With GC-MS analysis, eight compounds were identified in the extract. The major compounds were 9,12-octadecadienoic acid (Z, Z) (18.00%), *n*-hexadecanoic acid (17.60%), and squalene (4.12%), which are of reported medicinal properties. Genotoxicity results indicated that the extract markedly and significantly reduced DNA fragmentation in testicular and hepatic cells in mice. Haematological analysis showed that the extract significantly increased white blood cells (WBC), lymphocytes (LYMP), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), and significantly reduced packed cell volume (PCV), haemoglobin concentration (HB), and red blood cell (RBC) count. These results indicate that the *n*-hexane extract of *Olax subscorpioidea* leaves contain compounds that possess useful pharmacologic properties, and may reduce DNA damages as well as alter haematological indices.

**Keywords:** Toxicity, GC-MS, phytochemicals, genotoxicity, DNA, haematological indices.

**Introduction**

Plants have been used for ethnomedicinal purposes in the treatment of various illnesses since antiquity and they are widely used as alternative to orthodox medicines in modern times. In addition, documentation abounds providing scientific evidences attesting to the fact that extracts from different plants possess useful bioactivities and may be used in the management of different disease conditions. This may explain in part the reason herbal medicines are increasingly used and are recording huge sales around the world. As the use of herbal medicines continues to increase globally, concerns surrounding their safety are also increasingly recognized. In concomitant to their medicinal properties, herbal medicines—like the orthodox drugs—can exhibit toxicity to men. Toxicity is defined as the degree to which a chemical substance or a particular mixture of substances can damage an organism. Toxicity can occur on many different time-scales and can be classified as acute, sub-chronic and chronic. The toxic damages can be on a whole organism or its organs, tissues or cells and as a result toxicity can also be classified as: on whole organism (lethality), on organs (e.g. liver: hepatotoxicity, kidney: nephrotoxicity), on tissues (e.g. blood: haematoxicity), on cells (cytotoxicity). Toxicity tests are done to investigate, inter alia, the lethal, reproductive, developmental, immunological and hematological effects of a substance for human use.\(^5\) Cytotoxicity, the toxicity of a substance to cells, is important in toxicity investigation. Cell is the fundamental unit of life; the toxic effects at cellular level are the roots of those observe at tissue, organ or systemic level. Cytotoxicity tests are usually done to evaluate a substance for unwanted cytotoxic property or anticancer potential in targeting rapidly dividing cancer cells. Two of the hallmarks of cytotoxicity are necrosis and apoptosis and these can be triggered by damage to deoxyribonucleic acid (DNA) or other genetic materials.\(^6\) DNA contains the genetic instructions for the development and functions of a cell. Damage to DNA implies loss of the integrity of a cell which can lead to loss of cell functions or cell death and can as well initiate neoplasm and impairment of affected tissues or organs. The toxicity of a substance on DNA or other genetic material is described as genotoxicity. To estimate the genotoxic effects of a substance, the damage on DNA in cells exposed to such substance is assayed.\(^7\) The toxic effects of a substance on blood and its components can be described as haematoxicity. Blood performs many important functions within the body which includes; delivery of nutrients and oxygen to cells and tissues, removal of waste products from cells and tissues, immunological functions, messenger functions and regulation of core body temperature.\(^8\) These functions are dependent on appropriate balance of the components of the blood. A substance may interfere with blood leading to depletion or accretion of any of its

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components which may alter the blood’s biochemical properties and functions and have adverse effects. Therefore, investigation of the haematoxicity or haematological changes induced by a substance is important.

The medicinal or toxic properties of a plant are determined by its phytochemical constituents. Most phytochemicals are secondary metabolites and the most important bioactive ones include alkaloids, flavonoids, tannins, terpenoids, and glycosides among others. These groups of phytochemicals possess diverse bioactivities and offer insight into the biological effects of a plant. To identify specific phytochemical responsible for certain bioactivity it is important to probe the individual compounds. *Olia subscorpioidea* Oliv. belongs to the family Olacaceae. It is a tree or sometimes a many-stemmed shrub that grows up to 10 m high. *O. subscorpioidea* is widely found across Nigeria, Senegal, Zaire and Western Cameroun. It is called Ifon by the Yorubas, Atu-ogili by the Igbos and Gwaanom karmii by the Hausas in Nigeria.\(^9\) Ethnobotanical surveys have revealed that different parts of *O. subscorpioidea* is used for the management of diabetes mellitus,\(^15\) cancer,\(^16\) infectious diseases, mental illnesses,\(^12\) arthritis, rheumatism, and asthma.\(^11\) Also, researches have shown that the plant displayed antimicrobial,\(^14\) anti-ileuc,\(^13\) antinoceptive,\(^16\) anti-protease,\(^15\) and anti-depressant\(^16\) activities. The leaves\(^14\) and stem\(^14\) of the plant have been reported to contain classes of bioactive compounds; among them are tannins, alkaloids, saponins, flavonoids, and glycosides. *O. subscorpioidea* has a characteristic scent which is an indication that it contains volatile aroma compounds that can be extracted as essential oils by hydrodistillation or as concrete by solvent extraction with non-polar solvents. To the best of our search, there is no documentation on the volatile extract of *O. subscorpioidea*. Therefore, the present study is aimed at extracting and profiling the volatile and non-polar secondary metabolites of *O. subscorpioidea* leaves as well as investigating their genotoxic and haematological properties.

Materials and Methods

### Chemicals and Reagents

- n-Hexane (BDH), normal saline, phosphate buffer saline, tween 80, trichloroacetic acid, glacial acetic acid, sulphuric acid, diphenylamine, and all other reagents used were of analytical grade.

### Collection and Authentication of Plant Materials

Fresh leaves of *O. subscorpioidea* were collected from Sango-ota, Ogun State in April, 2017. The plant materials were authenticated by Mr Oyebanji at the Herbarium of the Department of Botany, University of Lagos, Akoka, where the voucher specimen (No. LUH: 7562) was deposited.

### Extraction of Plant Materials

The collected leaves of *O. subscorpioidea* were air dried under shade and the dried materials ground into fine powder. The pulverized plant material (300 g) was extracted — three times — by maceration with 1.5 L of n-hexane at room temperature for 72 h. The extracts were combined and concentrated in vacuo using rotary evaporator to obtain a semi-solid sticky mass (approximately 2.0%/w/w yield) herein referred to as *O. subscorpioidea* leaves n-hexane extract (OSLH).

### Analysis of Chemical Composition

#### Analysis of the chemical composition of the n-hexane extract of *O. subscorpioidea* leaves (OSLH)

The analysis of the chemical composition of the n-hexane extract of *O. subscorpioidea* leaves (OSLH) was done on GC-MS (Agilent Technologies) equipped with HP-5MS capillary column (30.0 x 0.25 mm x 0.25 μm; composed of 100% dimethyl poly-siloxane) and helium as the carrier gas at a flow rate of 2.0 mL/min. GC oven temperature was programmed as 45°C (5 min) to 325°C (10 min), at an increasing rate of 15°C/min. Total GC running time was 35 min. The temperature of the injector and the interface were set at 280°C and 350°C, respectively. Mass spectra were recorded on an electron ionization system with ionization energy of 70 eV, and an ion source temperature of 230°C. Identification of compounds was done by comparison of their spectra data with reference data in National Institute of Standards and Technology (NIST) library.

### Toxicity Studies

#### Experimental Animals

Male albino mice, average weight of 21 g, were obtained from animal facility centre, College of Medicine of the University of Lagos and used for this study. The animals were kept in a well-ventilated animal house, fed with standard mice diet and water *ad libitum* and acclimatized for 14 days prior to the experiment.

#### Experimental Protocols

Toxicity study was carried out according to OECD\(^9\) limit test (with some modifications) and was approved by the Health Research Ethics Committee of College of Medicine, University of Lagos with reference number; CMUL/HREC/03/18/341. The acclimatized animals were randomly split into three groups — 1, 2 and 3 — of three animals each.

- Groups 1 and 2, the control groups, were orally administered normal saline: 0.90% w/v of NaCl in water (negative control) and 3% tween 80-normal saline (vehicle control) respectively. Group 3, the test group, was orally administered 50 mg/kg dose of OSLH (in 3% tween 80-normal saline). The animals were observed for 48 h for behavioural variations. At the end of this period, blood samples were collected by retro-orbital puncture with capillary tube into EDTA bottles, the animals were sacrificed by cervical dislocation and their livers and testes were harvested for genotoxicity studies.

### Genotoxicity Studies

#### Qualitative Assessment of DNA Fragmentation by Gel Electrophoresis

Qualitative assessment of DNA fragmentation was carried out on agarose gel electrophoresis. Isolation of genomic DNA from the liver and testis samples was done according to Feiki et al.\(^26\) with some modifications. A portion (20 mg) of each organ was washed with 100 μL of phosphate buffer saline (PBS), followed by addition of 200 μL lysis buffer and 10 μL proteinase K, the resulting mixture was mixed in vortex mixer and heated in water bath at 55°C for 2 – 3 h. To this mixture, equal volumes (100 μL) of tris-EDTA (TE) buffer and 2% sodium dodecyl sulphate (SDS) were added, vortex mixed and then centrifuged at 12,000 g for 1 min to extract the DNA. Equal volume of extracted DNA (20 μL) was loaded on 2% agarose gel, stained with ethidium-bromide DNA dye and electrophoresed at 40 V for 2 h in X0.5 tris/acetate/EDTA (TAE) buffer. DNA fragments were visualised and photographed by exposing the gel to ultraviolet (UV) transillumination.

#### Estimation of Fragmented DNA by Diphenylamine (DPA) Assay

Estimation of DNA fragmentation was carried out according to the colorimetric DPA assay described by Gibb et al.\(^27\). A portion (30 mg) of each harvested organ was lysed in 0.5 mL lysis buffer and centrifuged at 10,000 r.p.m. (Eppendorf) for 20 min at 4°C. The pellets were resuspended in 0.5 mL of lysis buffer. To the pellets (P) and supernatant (S), 1.5 mL of 10% trichloroacetic acid (TCA) was added and mixed twice at 4°C for 10 min. Subsequently, to each sample, 2 mL of DPA solution was added and incubated at room temperature for 24 h. The absorbance of the samples was then taken at 600 nm and the proportion of fragmented DNA was calculated from the following expression:

\[
\% \text{ DNA fragmentation} = \frac{A_{\text{of frag. DNA}} \times 100}{A_{\text{of intact DNA}}} = \frac{A_{\text{of frag. DNA}}}{A_{\text{of intact DNA}}} \times A_{\text{of intact DNA}}
\]

where A is the absorbance of the sample.

### Assay of Haematological Parameters

The following haematological parameters, haemoglobin concentration (HB), red blood cell (RBC) count, white blood cell (WBC), packed cell volume (PCV), platelets (PLT), neutrophils (NEU), monocytes (MID), lymphocytes (LYMP), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were measured on an Auto Haematology Analyzer (MINDRAY BC-3200).

#### Statistical Analysis

All values are reported as the mean ± S.E.M. (standard error of mean). The values of the variables were analysed for statistically significant differences using the Student’s t-test on Microsoft Excel 2010® Data Analysis tools. Differences were considered significant at \(P < 0.05\).
Results and Discussion

There is increasing concern about the harmful effects of synthetic additives used in food and pharmaceutical industries and this has contributed to increasing interest in ingredients from natural sources as an alternative. The use of plants volatile and aromatic extracts as ingredients in foods, drinks, and cosmetics is gaining recognition. We herein present our findings from the investigation of the chemical, genotoxic and haematological properties of the volatile and non-polar secondary metabolites of O. subscorpioidea leaves.

GC-MS Analysis of Chemical Composition

The results of GC-MS analysis of the chemical composition of the n-hexane extract of O. subscorpioidea leaves (OSLH) are as presented in Table 1 and Figures 1 - 2, which show the identified secondary metabolites with their retention time, molecular formula, molecular weight, percentage composition, and structures. The most abundant compounds are 9,12-octadecadienoic acid (Z,Z)- (18.0%) and n-hexadecanoic acid (17.6%).

9,12-Octadecadienoic acid (Z,Z)- (linoleic acid), a compound made up of 18-carbon chain with 2-double bonds in cis configuration (cis-9,12), is a polyunsaturated essential fatty acid. Fatty acids have been reported to possess antimalarial potential to inhibit the fatty acid biosynthetic machinery of Plasmodium falciparum parasite.\textsuperscript{22} 9,12-Octadecadienoic acid (ZZ)- has been shown to inhibit Plasmodium berghei growth by 64% using the 4-day suppressive test.\textsuperscript{23} Linoleic acids with two conjugated double bonds (CLA: Conjugated linoleic acids) have been shown to suppress human tumor,\textsuperscript{24} to reduce metastasis of cancers to lung tissue\textsuperscript{25} and to possess antiatherogenic, antiangiogenic and immune modulating properties.\textsuperscript{25}

The leaves extract also contains n-hexadecanoic acid (17.6%), a fatty acid which is widely used in food and cosmetic industries. n-Hexadecanoic acid has shown anti-inflammatory property. Aparna et al,\textsuperscript{26} reported that it is a potent inhibitor of phospholipase A2, a pro-inflammatory enzyme. n-Hexadecanoic acid has also been reported to strongly boost metastasis in mouse models of human oral cancer.\textsuperscript{27} Based on indication that it may increase low-density lipoproteins (LDL) levels in the blood, consumption of n-hexadecanoic acid has been reported to increases the risk of developing cardiovascular diseases.\textsuperscript{28} Squalene, a triterpene and a biochemical precursor in the biosynthesis of steroids, was identified in the leaves. Due to its bio compatibility, squalene is used as an excipient in pharmaceutical formulations. Supplementation of squalene to mice has been reported to markedly increase cellular and non-specific immune functions in a dose-dependent manner and squalene is used in conjunction with surfactants in vaccines to stimulate immune response.\textsuperscript{29}

Octadecanoic acid, which was also identified, is widely used as surfactant and softening agent due to its bipolar nature and found wide application in cosmetic, soap, and detergent industries. Other compounds identified are long chain alkanes: nonacosane, and hentriacontane; an unsaturated aldehyde: 9,17-octadecadienal, (Z); and an ester: 7,10,13-hexadecatrien-1-carboxylic acid methyl ester which has unknown pharmacological property save hentriacontane which has been reported to possess anti-inflammatory property.\textsuperscript{30}

Genotoxicity Assays

The results of genotoxicity assays of n-hexane extract of O. subscorpioidea leaves (OSLH) are as presented in Figure 3 and Table 2, which show agarose gel electrophoresis and percent DNA fragmentations of livers and testes in OSLH treated and control groups. DNA fragmentation was detected in all the groups. Agarose gel electrophoresis (Figure 3) showed the formation of smear, indicating DNA laddering, which was greater in the testes than the livers in the OSLH treated and control groups. DNA laddering represents random DNA fragmentation, a characteristic feature of apoptosis or necrosis.\textsuperscript{31} which are binary events with consequential physiologic effects. Furthermore, the DNA fragmentation was determined quantitatively with diphenylamine (DPA) assay. The DPA assay revealed that the DNA fragmentations in hepatic and testicular cells were lower in the OSLH treated group (27.94 ± 0.41 and 49.74 ± 1.78, respectively) compared to the vehicle control group (45.80 ± 0.93 and 60.52 ± 8.49, respectively) (Table 2). These suggest that the n-hexane extract of O. subscorpioidea leaves effected marked reduction in DNA fragmentation in hepatic and testicular cells with compared to the vehicle control group and indicate that the extract could reduce DNA damage induced by physiologic stress. This may be associated to the presence of bioactive secondary metabolites in the extract, particularly, n-hexadecanoic acid which is abundant in the extract and has been documented to possess anticancer property.\textsuperscript{27}

Assays of Haematological Parameters

The results of the hematological study of n-hexane extract of O. subscorpioidea leaves in mice are as presented in Table 3. The results showed that the extract significantly reduced haemoglobin concentration (HB), packed cell volume (PCV), red blood cell (RBC) count, neutrophils (NEU), and monocytes (MID), and significantly increased white blood cell (WBC), lymphocytes (LYMP), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) compare to the vehicle control group. The reduction in HB and PCV mirrored the reduction in RBC which is implicative of anaemic effect.

![Figure 1: Total ion chromatogram (TIC) of n-hexane extract of O. subscorpioidea leaves (OSLH).](image-url)
Table 1: Chemical composition of n-hexane extract of *O. subscorpioidea* leaves (OSLH).

<table>
<thead>
<tr>
<th>S/N</th>
<th>Ret. time (min)</th>
<th>Name</th>
<th>Molecular formula</th>
<th>Molecular weight (g/mol)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>16.862</td>
<td><em>n</em>-Hexadecanoic acid</td>
<td>C_{16}H_{32}O_{2}</td>
<td>256.424</td>
<td>17.6</td>
</tr>
<tr>
<td>II</td>
<td>17.982</td>
<td>7,10,13-Hexadecatrienoic acid, methyl ester</td>
<td>C_{17}H_{32}O_{2}</td>
<td>264.043</td>
<td>0.84</td>
</tr>
<tr>
<td>III</td>
<td>18.927</td>
<td>9,17-Octadecadienal, (Z)-</td>
<td>C_{18}H_{32}O_{2}</td>
<td>264.465</td>
<td>2.87</td>
</tr>
<tr>
<td>IV</td>
<td>19.270</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
<td>C_{18}H_{32}O_{2}</td>
<td>280.446</td>
<td>18.0</td>
</tr>
<tr>
<td>V</td>
<td>19.689</td>
<td>Octadecanoic acid</td>
<td>C_{18}H_{32}O_{2}</td>
<td>284.477</td>
<td>4.05</td>
</tr>
<tr>
<td>VI</td>
<td>28.560</td>
<td>Squalene</td>
<td>C_{30}H_{50}</td>
<td>410.718</td>
<td>4.12</td>
</tr>
<tr>
<td>VII</td>
<td>29.617</td>
<td>Nonacosane</td>
<td>C_{29}H_{50}</td>
<td>408.787</td>
<td>5.54</td>
</tr>
<tr>
<td>VIII</td>
<td>32.155</td>
<td>Hentriacontane</td>
<td>C_{31}H_{64}</td>
<td>436.839</td>
<td>1.22</td>
</tr>
</tbody>
</table>

**Figure 2:** Structures of secondary metabolites identified in *n*-hexane extract of *O. subscorpioidea* leaves: *n*-Hexadecanoic acid (I), 7,10,13-Hexadecatrienoic acid, methyl ester (II), 9,17-Octadecadienal, (Z)- (III), 9,12-Octadecadienoic acid (Z,Z)- (IV), Octadecanoic acid (V), Squalene (VI), Nonacosane (VII), and Hentriacontane (VIII).

Elevated levels of WBC, PLT, and LYMP typically reflect the normal response of the immune system to infectious or inflammatory process. These cells are part of the immune system. They identify and eliminate pathogens or other invasive substances by contact or by phagocytosis. The elevated levels of these cells may be attributed to the presence of a constituent that triggers immune response in the *n*-hexane extract of *O. subscorpioidea* leaves. This is suspected to be squalene, which is reported to be used in conjunction with surfactants in vaccines to stimulate immune response. Although haematological index is not definitive, these findings suggest that the *n*-hexane extract of *O. subscorpioidea* leaves should be used with caution.

**Conclusion**

The secondary metabolites identified in the *n*-hexane extract of *O. subscorpioidea* leaves include 9,12-octadecadienoic acid (Z, Z)-; *n*-hexadecanoic acid; squalene, and hentriacontane, which are of documented pharmacological importance. Genotoxicity assays indicated that these metabolites demonstrated reduction in DNA fragmentation in both livers and testes of treated mice, which suggests that they may reduce DNA fragmentation or augment normal DNA repair system in these organs. Haematological assays revealed that the extract altered some haematological parameters in treated mice. These findings may give credence to some of the ethnomedicinal uses of *O. subscorpioidea* leaves and recommend caution in the medicinal use of this plant.

**Figure 3:** Agarose gel electrophoresis showing DNA fragmentation in mouse hepatic and testicular cells. Lane M: DNA molecular weight marker, Lane 1, 2, 3; liver of negative, vehicle control and OSLH treated mice respectively; Lane 4, 5, 6; testis of negative, vehicle control and OSLH treated mice respectively.
Table 2: DNA fragmentations of livers and testes of OSLH treated and control mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver DNA fragmentation (%)</th>
<th>Testis DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>56.07 ± 0.57</td>
<td>66.15 ± 2.22</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>49.74 ± 1.78</td>
<td>60.52 ± 8.49</td>
</tr>
<tr>
<td>OSLH</td>
<td>27.94 ± 0.41*</td>
<td>45.80 ± 0.93</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. (n=3). Values with asterisk (*) are significantly different (p < 0.05).

Table 3: Haematological indices of OSLH treated and control mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (10^6/L)</th>
<th>HB (g/dL)</th>
<th>PCV (%)</th>
<th>RBC (10^12/L)</th>
<th>PLT (10^3/mm)</th>
<th>NEU (%)</th>
<th>LYMP (%)</th>
<th>MID (%)</th>
<th>MCV (fL)</th>
<th>MCH (pg/cell)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>2.20 ± 0.03</td>
<td>14.60 ± 0.17</td>
<td>53.00 ± 0.61</td>
<td>9.30 ± 0.11</td>
<td>949.00 ± 10.90</td>
<td>44.00 ± 0.51</td>
<td>10.00 ± 0.12</td>
<td>46.00 ± 0.53</td>
<td>57.00 ± 0.66</td>
<td>15.60 ± 0.18</td>
<td>27.50 ± 0.32</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>2.30 ± 0.03</td>
<td>14.30 ± 0.17</td>
<td>52.10 ± 0.60</td>
<td>8.10 ± 0.09</td>
<td>938.00 ± 10.80</td>
<td>45.00 ± 0.51</td>
<td>9.00 ± 0.10</td>
<td>46.00 ± 0.53</td>
<td>64.20 ± 0.74</td>
<td>17.70 ± 0.20</td>
<td>27.40 ± 0.32</td>
</tr>
<tr>
<td>OSLH</td>
<td>3.40 ± 0.04*</td>
<td>13.60 ± 0.16*</td>
<td>43.00 ± 0.61*</td>
<td>6.20 ± 0.10*</td>
<td>969.00 ± 11.20</td>
<td>33.00 ± 0.38*</td>
<td>34.00 ± 0.39*</td>
<td>33.00 ± 0.38*</td>
<td>69.00 ± 0.80*</td>
<td>21.90 ± 0.25*</td>
<td>31.60 ± 0.36*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. (n=3). Values with asterisk (*) are significantly different (p < 0.05).

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References