Identification of HPV16 in suspected cases of cervical lesions and
docking Study of its L1 protein with some natural inhibitors.

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ABSTRACT

Background: The Human papillomavirus (HPV) causes sexually transmitted diseases. Among several types of HPV variants, HPV 16 is listed as a high-risk group, the primary cervical cancer etiologic agent, which causes life-threatening disease among women worldwide. The presence of L1, E6 and E7 encoded oncoproteins are largely responsible for virulence and pathogenicity that leads to cervical lesions. This menace is required to be curbed by designing an anti-cancerous drugs. The protein receptor-inhibitor interaction adopted using in silico analysis is very important in drug designing. It was the objective of this study to identify HPV16 isolates from suspected cases of cervical cancer at SH Sokoto and SYMH Birnin Kebbi hospitals and also to identify potent HPV16’s L1 protein inhibitor using in silico analysis of Echinacoside, curcumin and Cichoric acid against the viral protein.

Methods: A total of 140 cervical smear samples consisting of 21 low grade squamous intraepithelial lesion, 6 high grade lesion and 117 negative pap smears were collected. The samples were subjected for molecular detection using PCR targeting E6 and L1 genes of the virus. Positive samples were sequenced using Sanger sequencing platform. All the sequencing data were analysed using bioedit software while data generated for the molecular prevalence was statistically analyzed using Chi-square. A comprehensive HPV L1 protein homology model was designed to predict the L1 protein interaction mechanism with natural inhibitory molecules using a structural drug design approach. AutoDock Vina was used to carry out the molecular docking.

Results: Out of the 140 samples, 24 samples were positive for the PCR representing 16.7% molecular prevalence rate. There is statistically significant association between cyto-diagnoses and presence of HPV16 (P < 0.05). The highest prevalence rate of 12(50% of positive sample) was recorded among women between 30-39 years old. Docking analysis showed that the Chicoric acid components of Echinacea purpurae have strong binding affinity to the L1 protein of the HPV.

Conclusion: This study provides data on HPV 16 epidemiology in northern Nigeria, High-risk type 16 HPV variant was identified and also provides novel evidence for consideration on certain interacting residues, when synthesizing Anti-HPV compounds in the wet lab.

Keywords: HPV, Echinacea purpurae, Chicoric acid, Echinacoside, curcumin.
**Background**

Human papillomavirus is among the most prevalent infections transmitted during sexual activities which affects both male and female gender [1]. The size of the HPV genome is about 8kb, the genome is chromatinised, double stranded DNA (Deoxyribonucleic acid) which is enclosed in a 55 nm icosahedral capsid. HPV capsid is made up of two proteins, the L1 protein which is the major capsid protein and the L2 protein (the minor capsid protein). Each of the capsid consist of 360 monomers, which are arranged into 72 pentamers also known as capsomeres [2]. At early of HPV infection, the virion will first attach to heparin sulfate proteoglycans (HSPGs) situated in the cell surface or extracellular matrix [3]. L1 proteins will engage with HSPGs to cause conformational change in L1 and L2 proteins [4]. The host cell cyclophillin B facilitates the N-terminus to be expose, which will reveal a furin convertase cleavage site [5]. Following the conformational changes, the virion is associated with some non-HSPG receptors such as tetrasparins, annexin A2, growth factor receptor and integrins, to facilitate viral entry [6].
The virion enters the host through the endocytic route [6]. Human Papillomaviruses disease progression ranges from benign lesions to malignant [7]. The HPV types which are carcinogenic to the mucos membrane belongs to the genus alpha-papillomavirus, HPV types (HPV16 and 18), are the major cause of cancer of the cervix [8], they also cause vaginal, anal, oral, vulvar and penile cancers. The genus Alpha-papillomavirus also contains the benign mucosal HPV types that causes genital condylomas that are benign [9].

In developing countries HPV prevalence is higher, where asymptomatic infection is responsible for 44% of population [1]. A research was conducted in rural Nigeria, where 14.7 per cent of detectable high-risk HPV was registered, and representing two-thirds of the participants [10]. In north-eastern Nigeria, 48.7% of the participant are positive for HPV type 18, while HPV type 16 was 13.2%, and HPV type 31, 33 and 35 accounted for 18.5% together [11]. Making HPV type 18 the predominant HPV in North-eastern Nigeria. Also, the high risk HPV is predominant in South-western part of Nigeria [12]. In kano North-west of Nigeria about 76% of the women positive for HPV are either HPV type 16, 18 or both while 60.5% are co-infected with HPV 16 and 18 [13].

The infections caused by HPV could lead to an end terminal stage disease, so the availability of some certain medicinal plants which possess pharmacological potentials are implored as chemotherapeutic agent for infections. In order to explore natural drugs with lesser side effect and cost,
a natural compound Purple coneflower, is one among the plant reported to have active components such as chicoric acid, polysaccharides and echinacoside. This plant extract is known to stimulate immune response. The aqueous fractions of the stems, leaves, and flowers of Echinacea purpurea possess potent antiviral activity against HSV (Herpes Simplex Virus) type 1 and 2, and hemagglutinin of influenza virus. This activity was attributed to the plant extract components, polysaccharide and cichoric acid. A potent antiviral photosensitiser was seen in the ethyl acetate and ethanol soluble fractions of the plants stem and leaves [14]. Another molecular docking research in which one of the plants components, L-chicoric acid was docked against the protein HIV-1 (Human immunodeficiency virus type 1) integrase [15], shows a very good binding modes between the ligand and the viral integrase, this explains its reported potency which is consistent with the experimental data available [15].

In most part of northern Nigeria, the prevalence of HPV is unknown, and the infection have been poorly managed in the region in question, therefore the main objective of this study to identify HPV16 isolates from suspected cases of cervical lesion at Specialist Hospital (SH) Sokoto and Sir Yahaya Memorial Hospital (SYMH) Birnin Kebbi and also to identify potential HPV16’s L1 protein inhibitor using in silico analysis of Echinacoside, curcumin and Cichoric acid against the viral protein.
Methods

Study population
A total of 144 women with an average age of 33 years (range 20-60 years) participated in this study, population was recruited from patients attending Obstetrics and gynaecology units of SYMH Birnin Kebbi and SH Sokoto, Nigeria.

**Sample collection**

Cervical smear was collected from each of the participant by an application of standard procedure using sterilized speculum and swab (cyto-brush). The speculum was inserted through the vaginal orifice to allow visualization of the cervix. Cyto-brush was therefore placed into the endocervix and rotate in a circular fashion allowing collection of cellular smears from both ectocervix and endocervix (Squamocolumnar junction). The cyto-brush was removed, and its bristle was detached into the vial containing the fixative [16].

**Nomenclature**

This study adopted the following cytological classification: Negative; for normal cytology, low grade intraepithelial lesion, high grade intraepithelial lesion and carcinoma in situ; for malignancy.

**DNA extraction**

DNA from cervical samples were extracted using Viral Nucleic Acid Extraction kit II (Geneaid Biotech LTD, Taiwan). Extraction was carried out following the manufacturers’ instruction.

**Polymerase Chain Reaction**
The PCR was conducted using KOD-FX Neo (Toyobo, Japan) following manufacturer instructions as follows, each PCR mix of 50µL contained, buffer 25µL, 1.3 µL of forward and reverse primer (HPV16 Pr1 and HPV16 Pr2) and each, Deoxynucleoside triphosphate (DNTP) of 10µL, molecular grade water of 10 µL, DNA template 1.4 µL, KOD of 1 µL. the following PCR conditions were used, 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, final extension at 72°C for 5 minutes, then held for 16°C

**Sequencing**

The amplicons that showed desired band size at expected region for the target gene were sequence using sanger sequence method, sequences were aligned using BioEdit, and blasted on NCBI website, using the highly similar web tool.

**Statistical analysis**

Statistics analyses were performed using Chi-square with aid of an SPSS version 16.0, and data were presented in Tables and figures.

**Molecular Docking**

**Hardware and software**

All computational simulation studies were performed using (Intel Core i5-2430M) 6.00GB RAM with processor 2.40 GHz on windows7 operating system. Docking analysis was carried out using bioinformatics software such
as PyRx virtual screening software (AutoDock Vina) and the visualization of structure was carried out using Pymol molecular graphic system with the procedure summarised in Figure 1. Online resources were also used in this study.

**Ligand**

For this present study, ligands were downloaded from zinc12 (zinc.org), and saved in mol2 format, and subsequently converted into pdbqt format by autodock vina.

**Protein**

Pentamer structure of L1 protein of human papillomavirus (PDB Code: 2r5h) was retrieved from the protein data bank and saved. The file was consequently opened by double clicking the folder containing the protein structure which comes out in Pymol. The receptor was prepared by deleting protein and water via edit then delete. The protein was converted from the downloaded file format to pdbqt format which is the Vina format.

**Protein structure prediction**

Phyre 2 server was used in the prediction of the L1 protein of the human papillomavirus.

**Receptor-Ligand docking using PyRx virtual screening software**

AutoDock Vina was used to carry out the protein-ligand docking [17]. In order to perform protein-ligand docking, both the ligands and receptor (2r5h) were
converted from pdb files to pdbqt (protein data bank, partial charge Q and atom type T) files (Vina input file format). AutoDock Tools (ADT) was used to prepare L1 protein of the human papillomavirus by the complete addition of hydrogen atoms to the receptor’s carbon atoms. Non-polar hydrogen were added to the docked ligands. For Lamarckian genetic algorithm, a maximum number of $15 \times 10^5$ energy evaluations, 27,000 maximum generations, 0.02 gene mutation rate and 0.8 crossover rate were used [18]. Each ligands has had two hundred independent docking runs.
Figure 1: Algorithm of the performed docking analysis

Preparation and optimization of

Ligand was retrieved from Zinc12 (in mol2 format)  
Protein structure was downloaded from protein data

Vina was used to convert structure to

Binding affinity was calculated with

Result was exported to Pymol

Identification of binding site and interaction between  
Determination of bond length in Angstrom
Results

A self-administered questionnaire was given to participants, information on their sexual history was obtained. Table 1 shows that majority of the participants 64.1% have one sexual partner.

**TABLE 1: Socio-Cultural risk factors of the participants in relation to PCR result**

| Variables         | Values     | HPV 16 positive | HPV 16 negative | P value |
|-------------------|------------|-----------------|-----------------|---------|
| Number of sexual partners | One        | 18              | 105             |         |
|                    | Two        | 6               | 9               | `<0.05` |
|                    | Three or more | 0             | 6               |         |
| contraceptives    | Yes        | 9               | 42              |         |
|                    | No         | 15              | 78              | 0.816   |
| Smoking           | Yes        | 0               | 0               | a       |
|                    | No         | 24              | 120             |         |

Statistically significant associations are bolded (p `<0.05`), a- no statistic is computed because variable is constant.
The prevalence of HPV 16 is 16.7%. The cytological positive and negative results were compared with the PCR positive and negative result. The low grade squamous intraepithelial lesion (LGSIL) in this study was 21, with about 57% of the low-grade lesion been positive for HPV type 16 virus, none of the participants in this study has cancer, only 6 subjects showed cytology of high grade squamous intraepithelial lesion (HGSIL) and all been positive for HPV type 16. The P value of cytology-diagnosis in relation to presence of HPV16 is statistically significant (≤0.05) as shown in Table 2.

All the subjects that were positive for HPV type 16 complained of either genital itching, PV discharge or both symptoms, with genital itching (12) been most occurring symptoms among HPV positive subjects. The P value is statistically significant (≤0.05) as shown in table 2.

Table 2: Distribution of HPV 16 according to cytology and clinical symptoms

| Variables | Values   | HPV 16 positive | HPV 16 Negative | P value |
|-----------|----------|-----------------|-----------------|---------|
| Cytology  | Negative | 6               | 111             |         |
|           | LGSIL    | 12              | 9               | 0.05    |
|           | HGSIL    | 6               | 0               |         |
Carcinoma in situ

| Clinical symptoms | Genital itching | 15 | 27 |
|-------------------|-----------------|----|----|
|                   | PV discharge    | 3  | 24 |
| No complain       | 0               | 39 |    |
| Both genital      | 6               | 36 |    |

itching and PV discharge

Figure 2: Agarose gel showing polymerase chain reaction on amplified product of HPV 16 E6 gene. L-100 base pair (bp) DNA ladder,
A-positive control (band size=119), B, C and D- positive for HPV, E, F, G-negative for HPV

### Selected results of 200 independent run for the ligands chicoric acid, curcumin and Echinacoside with protein 2r5h

| Ligand                | Binding Affinity | rmsd/ub | rmsd/lb |
|-----------------------|------------------|---------|---------|
| 2r5h_zinc_33737268    | -8.7             | 0       | 0       |
| 2r5h_zinc_33737268    | -8.7             | 9.82    | 4.8     |
| 2r5h_zinc_33737268    | -8.6             | 6.992   | 3.943   |
| 2r5h_zinc_33737268    | -8.5             | 2.185   | 1.358   |
| 2r5h_zinc_33737268    | -8.5             | 9.631   | 4.566   |
| 2r5h_zinc_33737268    | -8.5             | 5.281   | 3.91    |
| 2r5h_zinc_33737268    | -8.4             | 2.372   | 1.606   |
| 2r5h_zinc_33737268    | -8.4             | 3.345   | 2.029   |
| 2r5h_zinc_33737268    | -8.3             | 8.924   | 4.438   |
| 2r5h_zinc_899824      | -6.8             | 0       | 0       |
| 2r5h_zinc_899824      | -6.6             | 9.031   | 3.149   |
| Protein ID               | Value 1 | Value 2 | Value 3 |
|-------------------------|---------|---------|---------|
| 2r5h_zinc_899824        | -6.6    | 7.636   | 1.819   |
| 2r5h_zinc_899824        | -6.5    | 8.764   | 2.993   |
| 2r5h_zinc_899824        | -6.4    | 6.335   | 2.528   |
| 2r5h_zinc_899824        | -6.4    | 8.154   | 3.935   |
| 2r5h_zinc_899824        | -6.4    | 6.11    | 2.708   |
| 2r5h_zinc_899824        | -6.4    | 5.495   | 3.166   |
| 2r5h_zinc_899824        | -6.3    | 5.651   | 2.585   |
| 2r5h_zinc_95098864      | -8.6    | 0       | 0       |
| 2r5h_zinc_95098864      | -8.3    | 8.723   | 3.508   |
| 2r5h_zinc_95098864      | -8.3    | 7.656   | 3.725   |
| 2r5h_zinc_95098864      | -8.2    | 3.277   | 2.392   |
| 2r5h_zinc_95098864      | -8.2    | 4.709   | 3.501   |
| 2r5h_zinc_95098864      | -8.2    | 7.908   | 3.489   |
Figure 3: Crystal structure of HPV 16 L1 pentamer and Ligands A: cartoon structure of HPV 16 L1 pentamer (2R5H), B: Ligand Cicoric acid in receptor pocket, C: Ligand Echinacoside in receptor pocket showing amino acid residues, D: ligand chicoric acid docked against pentamer structure of major capsid protein L1 of HPV showing polar contact and bond length.
Discussions

HPV type 16 has been the major cause of cervical lesion in Africa, it causes 49% of cervical cancer in the continent, higher than any other HPV serotype. HPV type 16 is responsible for 70 percent of all cervical cancer alongside with type 18 [19]. Of the 144 participants recruited in this study 16.9% were positive for HPV type 16, which is slightly higher than the study carried out in Kano, Nigeria where they reported prevalence of 15.8% for HPV type 16, though their study recruited lesser subjects (50) than this study [13]. A study carried out in Lagos, Nigeria, which they reported HPV type 16 to be 46.9%, this is not in accordance with the present study [20], another study reported 23.5% [21], while in a study carried out in the south western Nigeria HPV 16 prevalence (3.5%) was very low than the one in this study [22].

The low grade squamous intraepithelial lesion (LGSIL) in this study was 21, with about 57% of the low-grade lesion been positive for HPV type 16 virus, none of the participants in this study has cancer, only 6 subjects showed cytology of high grade squamous intraepithelial lesion (HGSIL) and all been positive for HPV type 16. The P value is statistically significant (‘0.05). This study shows there is an association between infection with HPV 16 and cervical lesions, this is consistent with a study where the strength of the association was even demonstrated with odd ratio of 182, showing a strong association between HPV 16 and cervical lesions [23]. While another study also showed HPV 16 is a strong risk factor for cervical lesion [21].
Symptoms such as genital itching, vaginal discharge (also known PV discharge), post-coital bleeding, are the most common symptoms seen in women with cervical abnormalities and vaginal infections. There was no complain of post-coital bleeding among the participants in this study, but all the subjects that were positive for HPV type 16 complained of either genital itching, PV discharge or both symptoms, with genital itching (12) been most occurring symptoms among HPV positive subjects. The P value is statistically significant (˂0.05). This is inconsistent with a study were they reported no association between infection with high risk HPV types and virginal problems (Itching, odor, and discharge) with P value of 0.14 [24]. though the symptoms can also be seen in other vaginal infections which are not tested for in this study, this might be the reason for the reported data not conforming with that of this study.

The use of oral contraceptives and cervical abnormalities have been closely associated. This study is highlighting presence of the virus that causes cervical abnormalities and its risk factors, so it is important to understand the association between HPV infections and contraceptive usage. About 35% of the participants in this study are on contraceptive, and 45 of the 51 subjects on contraceptives are negative for HPV. The P value is statistically insignificant (P=.815).

Molecular docking studies were carried out between the targets (pentamer structure of major capsid protein L1 of HPV type 16) which has 3 chains, and
its inhibitors (chicoric acid, echinacoside, curcumin). All the compounds were found to inhibit strongly by completely occupying the active sites in the target protein (2r5h), in this study the inhibitors is occupied within the AA 50-60 of the L1 protein, which is situated in the DE-Loop of the HPV L1 protein. Most of the ligands were found to be having polar contact with the receptor (2r5h). The best two docking results were zinc_33737268 (chicoric acid) with a binding energy of -8.7 kcal/mol which is lower than the binding energy reported for Withaferin A docked against HPV E6 protein in a study by Kumar et al. (2014), though in the same study carrageenan showed a much lower binding energy when docked against E6 protein, but the present study targeted the L1 protein which is a more conserved region, the L1 also has the heparin binding site in its loop, which plays important role in high risk HPV infection [25]. However many HSPG-mimicking compound have failed clinical phase III trials, which is explained to be due to dilution of this antiviral agent by body fluid, probably leading to loss of infectious particle [26]. The chicoric acid shows polar interactions with Ala139, Leu126, Asp127, Lys 125, Asn 124, Arg 144, Ala 134, Tyr355, Lys 356, and Gly256 of the target protein. With bond length (2.50 Å and 3.70 Å). Ligand zinc_95098864 with binding score of -8.6 kcal/mol forms bonds (1.20 Å, 1.52 Å) with Lys 125, Asn 124, Thr 226, Ser 227, Leu 275, Leu 222, Gly 183, Cys 185, Ala 264 and Pro 182.

**Conclusion**

Numerous researches are currently ongoing in order to identify promising therapeutic agent for the management of HPV associated diseases,
advancement in molecular modelling and bioinformatics are very important in validating those therapeutic agents using *in silico* analysis. This study provides data on some interacting residues for consideration, when synthesizing Anti-HPV compounds in the wet lab.

**List of abbreviation**

HPV – Human papillomavirus

SH – Specialist Hospital

SYMH – Sir Yahaya memorial Hospital

PCR – Polymerase chain reaction

DNA – Deoxyribonucleic acid

HSPG – Heparan Sulfate Proteoglycan
HSV - Herpes Simplex Virus

HIV-1 - Human immunodeficiency virus type 1

DNTP - Deoxynucleoside triphosphate

NCBI - National Centre for Biotechnology Information

SPSS - Statistical Package for the Social Sciences

PDBQT - Protein data bank, partial charge Q and atom type T

PDB - Protein data bank

LGSIL - Low grade squamous intraepithelial lesion

HGSIL - High grade squamous intraepithelial lesion

Declaration

Ethics Approval and consent to participate

Before commencement of sample collection an ethical approval was obtained from Kebbi State Ministry of Health Ethical Committee with reference number: SMOH/42/S/4675 and SH Sokoto, Hospital Ethics and Research Committee with reference number: SHS/SUB133/vol.1. The subjects whose signatures were obtained from the informed consent form participated in the
research. The research has been reviewed and accepted by Bayero University Kano, Thesis review Board.

**Consent for publication**

Not Applicable

**Availability of Data and Material**

Not applicable

**Competing interests**

The authors do not claim any competitive interests.

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**Authors’ contribution**

Concept of the research; BJA, AIA. Data collection: YL, DAB, KIM, AS, Data analysis; YL. Methodology: BJA, AIA, YL. Research administrator; KAS. Supervision: BJA, AIA. Editing and Reviewing: BJA, AIA.

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Figure Legends

Figure 1: Algorithm of the performed docking analysis

Figure 2: Agarose gel showing polymerase chain reaction on amplified product of HPV 16 E6 gene. L-100 base pair (bp) DNA ladder, A-positive control (band size=119), B, C and D- positive for HPV, E, F, G- negative for HPV

Figure 3: Crystal structure of HPV 16 L1 pentamer and Ligands A: cartoon structure of HPV 16 L1 pentamer (2R5H), B: Ligand Cicoric acid in receptor pocket, C: Ligand Echinacoside in receptor pocket showing amino acid residues, D: ligand chicoric acid docked against pentamer structure of major capsid protein L1 of HPV showing polar contact and bond length