

<http://dx.doi.org/10.48198/NJPAS/21.A19>

In silico Study of the Interactions between Schiff Base Polyphenols and HPV 16 E6/E6AP/p53 complex

Jeremiah I. Ogah,^{1*} Olatunji M. Kolawole,¹ Steven O. Oguntoye² and M.M. Suleiman^{1,3}

Page | 3973

¹Infectious Diseases and Environmental Health Research Group (IDEHRG), Department of Microbiology, University of Ilorin.

²Department of Chemistry, University of Ilorin.

³Department of Biological Sciences, Summit University, Offa.

ABSTRACT

The rise in the incidence of cervical cancer globally has accentuate attention to the potential role of polyphenols as anticancer agents. Different studies have demonstrated the role of some polyphenols in altering Human Papillomavirus (HPV) carcinogenesis. Thus, this study was aimed at establishing the potentials of Schiff-based polyphenols from imesatin and satin as anticancer agents through *in silico* analysis. The polyphenols were synthesized and characterized using elemental analyses, spectroscopic analyses, UV-visible, Infrared, and Nuclear Magnetic Resonance (¹H NMR and ¹³C, NMR). Molecular docking study of the polyphenols was carried out using Auto Dock Vina. The oncogenic E6 protein structure of HPV 16 was obtained from the protein bank (ID: 4XR8). The E6 proteins were prepared using AutoDock tools. Water molecules were removed from the protein molecules while hydrogen atoms were added. Also, the structures of Curcumin and Isomericitritin were obtained from PubChem. Results showed that three different Schiff based polyphenols were obtained from the synthesis; 3-(2',4'-dimethoxy benzylidene hydrazono) indoline-2-one (DMBH), 3-(2'-hydroxy-4'-methoxy benzylidene hydrazono) indoline-2-one (HMBD), and 3-((4-4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl)imino) indoline-2-one (DMBP). Higher ability of the docked polyphenols to bind to the E6/E6AP/p53 complex when compared to Curcumin was revealed. Also, results showed that the binding energy of Curcumin and Isomericitritin were -7.1kcal/mol and -8.4kcal/mol respectively while that of the polyphenols ranged from -7.4kcal/mol to -7.9kcal/mol. The molecular docking results of the polyphenols used in this study further confirm their potentials as strong anti-cancer agents.

Keywords: Cancer, molecular docking, polyphenols, HPV

Introduction

The incidence of cervical cancer has been on the increase in Nigeria, with more cancer deaths ascribed to it than any other type of cancer in Nigeria apart from breast cancer (Ogah *et al.*, 2019). Cervical cancer occurrence is frequently associated with the proliferation of E6 and E7

oncoproteins that are present in high-risk Human Papillomavirus (HPV). When HPV DNA enters into the host genome, the proteins E6 and E7 are produced from the resultant DNA. E6 binds and degrades p53 (a tumor suppressor gene), while E7 binds and degrades retinoblastoma (Rb) which is a

Corresponding Author: Jeremiah Ogah

Infectious Diseases and Environmental Health Research Group (IDEHRG), Department of Microbiology, University of Ilorin. Phone: +2348134144424 Email: Ogahij@yahoo.com



tumor suppressor gene (Doorbar *et al.*, 2015). The degradation of p53 and Rb by E6 and E7 oncoproteins respectively allows for cells to keep replicating without any check. This eventually leads to cancer progression. Retinoblastoma normally keeps the cell from growing too fast or responding to growth stimulators. This inhibitory factor is now lost. Without these two mechanisms to slow down cell growth and prevent mutation, malignant transformation occurs. Thus, any system or mechanism that will be applied in the treatment of HPV infection and cervical cancer, must be able to suppress the activities of E6 and E7 oncogenes (Paulraj *et al.*, 2015).

With a plethora of microbial infections plaguing man, ranging from viral (Kolawole *et al.*, 2017) to fungi infections (Durowade *et al.*, 2008), there is a constant search for bioactive compounds with capacity to impede the activities of disease-causing organisms. Polyphenols have been under extensive studies recently because of their potentials as anticancer agents. Mishra *et al.*, (2015) in their study of polyphenols showed that the widely known curcumin was able to activate cell apoptosis, restoring p53 and pRb when curcumin was used to treat cervical cancer cells. A significant number of reports have been able to show the importance of some widely known bioactive compounds as potential anticancer agents (Rodriguez-Mateos *et al.*, 2014; Paulraj *et al.*, 2015; Zaman *et al.*, 2016).

The potentials of bioactive components of plants as well as polyphenols, in general, to be used as anticancer agents could be evaluated through *in silico* studies (molecular docking). Molecular docking is a setup involving *in silico* structure-based techniques broadly utilized in drug discovery processes. Molecular docking utilizes the use of novel compounds with potential therapeutic effects to predict the interactions at sub-atomic levels between the potential therapeutic agents and their target items (Pinzi and Rastelli, 2019). Molecular docking can also be referred to as virtual chemistry. Its convenience, cost, reduction in laboratory time

for actual synthesis makes it a valuable tool in the drug discovery and development industry (Stefaniu, 2019). Thus, this study was aimed at utilizing molecular docking tools to predict potential interactions between polyphenols and E6 oncoprotein of HPV 16.

Materials and Methods

Molecular docking study of the polyphenols was carried out using Auto Dock Vina (Trott and Olson, 2010). The oncogenic E6 protein structure of HPV 16 was obtained from the protein bank (ID: 4XR8). The E6 proteins were prepared using AutoDock tools. Water molecules were removed from the protein molecules while hydrogen atoms were added.

3D Structures of 3-((4-4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl)imino)indoline-2-one (DMBP). 3-(2'-hydroxy-4'-methoxybenzylidenehydrazono) indoline-2-one (HMBD) and 3-(2',4'-dimethoxybenzylidenehydrazono) indoline-2-one (DMBH) were designed using ChemDraw software. Also, the structures of Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione) and Isomericitrin (5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-(3,4,5-trihydroxy phenyl) chromen-4-one) were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>). The ligand molecules were optimized using Discovery Studio Visualizer. Molecular interactions between Proteins and ligands with the highest binding affinity were viewed with discovery studio. The structure of the polyphenols used is shown in figure 1-3;

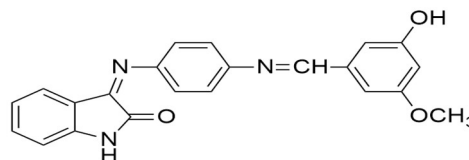


Figure 1: 3-((4-4'-((2'', 4''-dimethoxy benzylidene amino)benzyl)phenyl)imino)indoline-2-one (DMBP)

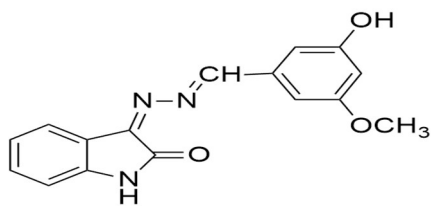


Figure 2: 3-(2'-hydroxy-4'-methoxybenzylidenehydrazono)indoline-2-one (HMBD)

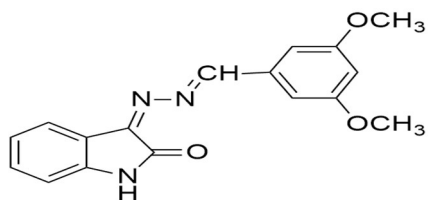


Figure 3: 3-(2',4'-dimethoxybenzylidenehydrazono)indoline-2-one (DMBH)

Results

Results from Table 1 shows that Curcumin has a binding score of -7.1 kcal/mol, with Isomericitritin having a binding score of -8.4 kcal/mol. Furthermore, results showed that 3-(2',4'-dimethoxybenzylidenehydrazono) indoline-2-one (DMBH), 3-(2'-hydroxy-4'-methoxy benzylidene hydrazono) indoline-2-one (HMBD) and 3-((4-4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl)imino) indoline-2-one (DMBP) had binding scores of -7.4 kcal/mol, -7.4 kcal/mol and -7.9 kcal/mol respectively (Table 1).

Figure 4 describes the 3D and 2D interaction between Curcumin and HPV 16 E6 oncoprotein. Results from Figure 4.10 shows that 6 different types of bonds existed between the Curcumin molecules and HPV 16 E6 oncoprotein. Figure 5 describes the 3D and 2D interaction between 3-(2',4'-dimethoxy benzylidene hydrazono) indoline-2-one (DMBH) and HPV 16 E6 Oncoprotein. Results from Figure 5 shows that that 7 different types of bonds existed between the DMBH molecules and HPV 16 E6 oncoprotein. Figure 6 describes the 3D and 2D interaction between 3-(2'-hydroxy-4'-methoxy benzylidene hydrazono) indoline-2-one (HMBD) and HPV 16 E6

Oncoprotein. Figure 7 describes the 3D and 2D interaction between 3-((4-4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl)imino) indoline-2-one (DMBP) and HPV 16 E6 Oncoprotein.

Table 1: Molecular docking of HPV 16 E6 Oncoprotein

Compounds	ΔG Energy (kcal/mol)
Curcumin	-7.1
Isomericitritin	-8.4
3-(2',4'-dimethoxybenzylidene hydrazono)indoline-2-one (DMBH)	-7.4
3-(2'-hydroxy-4'-methoxybenzylidenehydrazono)indoline-2-one (HMBD)	-7.4
3-((4-4'-((2'',4''-dimethoxy benzylidene amino) benzyl) phenyl)imino) indoline-2-one (DMBP)	-7.9

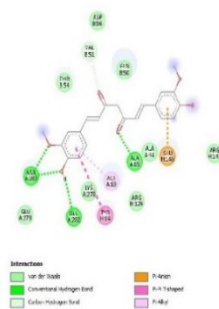
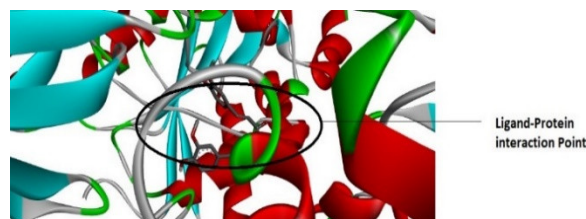
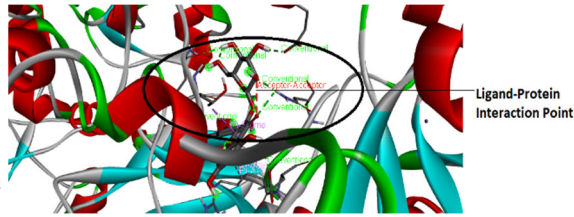
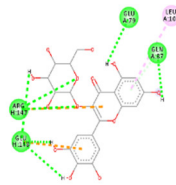


Figure 4: 3D and 2D interaction between Curcumin and HPV 16 E6 Oncoprotein

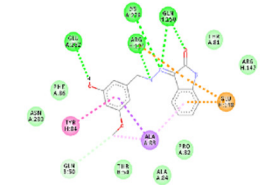
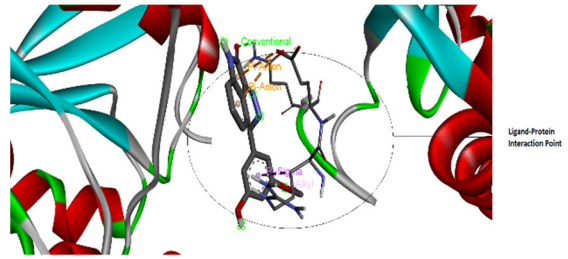


Page | 3976



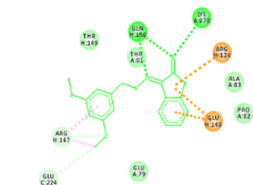
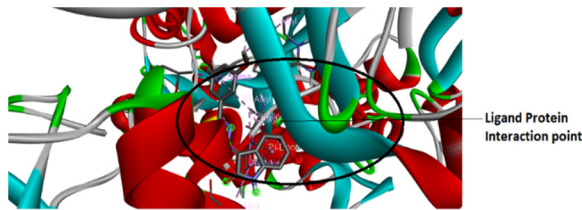
Interactions
 Conventional Hydrogen Bond
 H-Cation
 Pi-Anion
 Pi-Conjugated Hydrogen Bond
 Pi-Alkyl

Figure 5: 3D and 2D interaction between Isomericitrin and HPV 16 E6 Oncoprotein



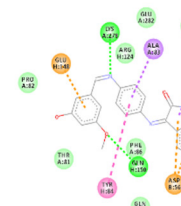
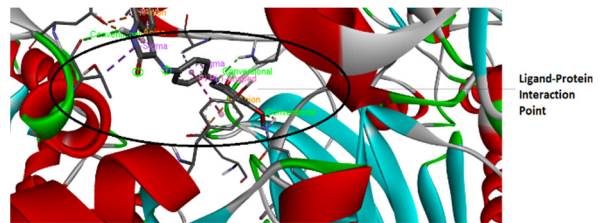
Interactions
 Van der Waals
 Conventional Hydrogen Bond
 Cation-Hydrogen Bond
 H-Cation
 Pi-Anion
 Pi-Stacked
 Pi-Alkyl
 Pi-Stacked
 Pi-Alkyl

Figure 7: 3D and 2D interaction between 3-(2'-hydroxy-4'-methoxy benzylidene hydrazono) indoline-2-one (HMBD) and HPV 16 E6 Oncoprotein



Interactions
 Van der Waals
 Conventional Hydrogen Bond
 Carbon Hydrogen Bond
 H-Cation
 Pi-Anion
 Alkyl
 Pi-Alkyl

Figure 6: 3D and 2D interaction between 3-(2',4'-dimethoxy benzylidene hydrazono) indoline-2-one (DMBH) and HPV 16 E6 Oncoprotein



Interactions
 Van der Waals
 Conventional Hydrogen Bond
 Pi-Anion
 Pi-Stacked
 Pi-Alkyl
 Pi-Stacked
 Pi-Alkyl

Figure 8: 3D and 2D interaction between 3-((4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl) imino)indoline-2-one (DMBP) and HPV 16 E6 Oncoprotein

Discussion

To further establish the capacity of the polyphenols 3-(2',4'-dimethoxy benzylidenehydrazono) indoline-2-one (DMBH), 3-(2'-hydroxy-4'-methoxy benzylidene hydrazono)indoline-2-

one (HMBD) and 3-((4'-((2'', 4''-dimethoxy benzylidene amino) benzyl) phenyl)imino)indoline-2-one (DMBP) as drugs in the treatment of cervical cancer, molecular docking which has the ability to determine the binding energy of

interacting molecules was used. These polyphenols which were prepared as ligands were docked against HPV 16 E6 oncoprotein. Furthermore, to create a reference point, popular bioactive compound Curcumin as well as Isomericitrin a bioactive compound found in *Bridelia ferruginea* (Afolayan *et al.*, 2019) were also docked against HPV 16 E6 oncoprotein. Molecular docking was used because of its imperative role in the design of drugs (Ferreira *et al.*, 2015).

During molecular docking, the binding energy is released when a drug molecule associates with a target, leading to a lowering of the overall energy of the complex (Monika *et al.*, 2010). The release in binding energy also compensates for any transformation of the ligand from its energy minimum to its bound conformation with the protein. Thus, the greater the energy released on the binding of a ligand to the protein, the lower the energy of the complex, leading to a higher propensity of the ligand to associate with that protein (Ferreira *et al.*, 2015).

Results from this study show that the binding energy of Curcumin at -7.1kcal/mol was higher than that recorded for DMBH, HMBD, and DMBP at -7.4kcal/mol, -7.4kcal/mol, and -7.9kcal/mol respectively (Table 1). This indicates that DMBH, HMBD, and DMBP are more effective against HPV 16 E6 oncoprotein when compared with Curcumin. Their lower binding energy is indicative of higher stability of their ligand when compared to that of Curcumin. The lower binding energy recorded for these polyphenols means their ligand would bound more spontaneously consuming less energy (Ferreira *et al.*, 2015). Thus, it is assumed that if you get a lower binding energy value for a particular molecule during docking simulation it means that your selected molecule binds appropriately with the biological molecule and vice versa. Low binding energy having dock

conformer has greater stability and vice versa (Monika *et al.*, 2010).

The HPV 16 E6 oncoprotein used for this docking is an intricate compound consisting of the E6/E6AP/p53 complex. This complex is a necessity in initiating HPV carcinogenesis. For HPV-induced cancer to occur, this complex has to bind with p53 a known tumour suppressor gene (Alba *et al.*, 2009). For this binding to proceed, E6 binds itself to a leucine-rich sequence present within the ligase E6AP (Martinez-Zapien *et al.*, 2016). Thus, potential drugs against cervical cancer focus on blocking the ability of this binding to occur, by forming a wedge between this complex and p53. This was successfully achieved in this study, as the conformational structures of the polyphenols used in this study showed that these polyphenols have a high binding ability to the E6/E6AP/p53 complex. Martinez-Zapien *et al.* (2016) stated that “mutagenesis of critical positions at the E6–p53 interface disrupts p53 degradation”. This further explains the need to continuously look for compounds that can actively bind with E6 oncoprotein, preventing it from binding with p53 and thus, altering HPV carcinogenesis (Huibregtse *et al.*, 1993).

Results from molecular docking showed that Curcumin and Isomericitrin had sufficient binding affinity to the HPV 16 E6 oncoprotein (Table 1). The exciting result from this study is the higher ability of the docked polyphenols to bind to HPV 16 E6 oncoprotein when compared to Curcumin which is a known, anticancer agent. Curcumin has been shown to have the ability to suppresses HPV oncoproteins, while restoring restores p53 and Rb (Maher *et al.*, 2011; Mamgain *et al.*, 2015). Furthermore, results showed that Isomericitrin had the lowest docking score -8.4kcal/mol (Table 1). This could account for the strong apoptotic activities of the Iron nanoparticles from *Bridelia ferruginea* described earlier in this study. While there is sparse literature on the biological

activities of Isomericitrin, it is well documented that it is an important bioactive compound found in *Bridelia ferruginea* (Afolayan *et al.*, 2019).

The potentials of these polyphenols to be used as anticancer agents as demonstrated in this study is backed by different molecular docking studies. Muthukala *et al.* (2015) in their *in-silico* study of Quercetin against Hela cells showed potential inhibitory properties of Quercetin against cervical cancer cells. Similarly, Mamgain *et al.* (2015) using molecular docking, showed that natural compounds had an inhibitory effect against HPV16 E6 oncoprotein. These reports were similar to what was obtained in this study using polyphenols. Also, Kumar *et al.* (2015) in their molecular docking study of natural inhibitors against E6 oncoproteins from HPV 18, showed sufficient binding activities between these inhibitors and the oncoprotein. Furthermore, Prasasty *et al.* (2017) in their *in-silico* study of the effect of natural inhibitors against HPV (16, 18, and 52) oncoproteins showed found the highest binding energy to interact with E6 HPV-16 was daphnoretin (-7.8 kcal/mol), HPV-18 and 52 was β -cryptoxanthin -7.8 kcal/mol and -8.4 kcal/mol. These binding scores are in the range of scores obtained in this study (-7.1 kcal/mol to -8.4 kcal/mol).

The binding forces which existed between the ligands and proteins contributed to the lower binding energy obtained in this study. Covalent bonding which existed between these molecules is of particular interest, because of the high binding energy it possesses (Kalgutkar and Dalvie, 2012; Doane and Burda, 2013; Pommier, 2013). Drugs with covalent interactions are known to have a longer-lasting effect on cells (Kumalo *et al.*, 2015). The covalent linkage existing between the molecules is very important firstly due to the role covalent bonding plays in the persistence of drugs in the human system (Kumalo *et al.*, 2015) and secondly due to the role covalent bonding plays in the mechanism of action of topoisomerase. DNA

topoisomerases usually form covalent intermediates, wherein tyrosyl residue is covalently linked to DNA phosphoryl (Schoeffler and Berge -r, 2008). Thus, covalent linkages are critically considered in the design of anti-tumor and antimicrobial drugs (Zhang *et al.*, 2017). About 33% of the covalent drugs available are anti-infectives (used majorly as β -lactam drugs), 15% are employed in the treatment of gastrointestinal disorders, 20% are often used in cancer treatment, and with 15% of these covalent drugs available used in the treatment of cases of Central Nervous System (CNS) related ailments (Singh *et al.*, 2011). The molecular docking results of the polyphenols used in this study further confirm their potentials as strong anti-cancer agents.

Competing interests

Authors have declared that no competing interests exist.

References

- Afolayan, M., Srivedavyasari, R., Asekun, O. T., Familoni, O. B. and Ross, S. A. (2019). Chemical and biological studies on *Bridelia ferruginea* grown in Nigeria. *Natural product research*, 33(2):287–291.
- Alba, A., Cararach, M. and Rodríguez-Cerdeira, C. (2009). The Human Papillomavirus (HPV) in Human Pathology: Description, Pathogenesis, Oncogenic Role, Epidemiology and Detection Techniques. *The Open Dermatology Journal*, 3:90-102.
- Doane, T. and Burda, C. (2013). Nanoparticle mediated non-covalent drug delivery. *Advanced Drug Delivery Reviews*, 65:607–621.
- Doorbar, J., Egawa, N., Griffin, H., Kranjec, C. and Murakami, I. (2015). Human papillomavirus molecular biology and disease association. *Reviews in medical virology*, 25(1): 2–23.

- Durowade, K.A., Kolawole, O.M., Uddin II, R.O. and Enobun, K.I. (2008). Isolation of ascomycetous fungi from a tertiary institution campus soil. *Journal of Applied Sciences and Environmental Management*, 11 (4)
- Ferreira, L.G., dos Santos, R.N., Oliva, G. and Andricopulo, A.D. (2015). Molecular Docking and Structure-Based Drug Design Strategies. *Molecules*, 20:13384-13421.
- Huibregtse, J.M., Scheffner, M. and Howley, P.M. (1993). Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Molecular and Cellular Biology*, 13:4918–4927.
- Kalgutkar, A.S. and Dalvie, D.K. (2012). Drug discovery for a new generation of covalent drugs. *Expert Opinion on Drug Discovery*, 7:561–581.
- Kolawole, O.M., Seriki, A.A., Irekola, A.A., Bello, K.E., Adeyemi, O.O. (2017). Dengue virus and malaria concurrent infection among febrile subjects within Ilorin metropolis, Nigeria. *Journal of medical virology*, 89 (8):1347-1353
- Kumalo, H.M., Bhakat, S and Soliman, M.E.S. (2015). Theory and Applications of Covalent Docking in Drug Discovery: Merits and Pitfalls. *Molecules*, 20:1984-2000.
- Kumar, S., Jena, L., Sahoo, M., Kakde, M., Daf, S. and Varma, A.K. (2015). In Silico Docking to Explicate Interface between Plant-Originated Inhibitors and E6 Oncogenic Protein of Highly Threatening Human Papillomavirus 18. *Genomics & informatics*, 13(2):60–67.
- Maher, D. M., Bell, M. C., O'Donnell, E. A., Gupta, B. K., Jaggi, M. and Chauhan, S.C. (2011). Curcumin suppresses human papillomavirus oncoproteins, restores p53, Rb, and PTPN13 proteins and inhibits benzo[a]pyrene-induced upregulation of HPV E7. *Molecular carcinogenesis*, 50(1):47–57.
- Mamgain, S., Sharma, P., Pathak, R.K. and Baunthiyal, M. (2015). Computer aided screening of natural compounds targeting the E6 protein of HPV using molecular docking. *Bioinformation*, 11:236-242.
- Martinez-Zapien, D., Ruiz, F. X., Poirson, J., Mitschler, A., Ramirez, J., Forster, A., Cousido-Siah, A., Masson, M., Vande Pol, S., Podjarny, A., Travé, G. and Zanier, K. (2016). Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53. *Nature*, 529 (7587):541–545
- Mishra, A., Kumar, R., Tyagi, A., Kohaar, I., Hedau, S., Bharti, A. C., Sarker, S., Dey, D., Saluja, D. and Das, B. (2015). Curcumin modulates cellular AP-1, NF-kB, and HPV16 E6 proteins in oral cancer. *E-cancer medical science*, 9:525.
- Monika, G., Puna, G., Sarbjot, S. and Gupta, G.D. (2010). An Overview on Molecular Docking. *International Journal of Drug Development and Research*, 2(2):219-231.
- Muthukala, B., Sivakumari, K. and Ashok, K. (2015). In silico docking of Quercetin compound against the Hela cell line proteins. *International Journal of Current Pharmaceutical Research*, 7:13-16
- Ogah, J.I., Kolawole, O.M., & Awelimobor, D. (2019). High Risk Human Papillomavirus (HPV) Common among a Cohort of Women with Female Genital Mutilation. *African Health Sciences*; 19(4):2985-2992

- Paulraj, F., Abas, F., Lajis, N. H., Othman, I., Hassan, S. S., & Naidu, R. (2015). The Curcumin Analogue 1,5-Bis(2-hydroxyphenyl)-1,4-pentadiene-3-one Induces Apoptosis and Downregulates E6 and E7 Oncogene Expression in HPV16 and HPV18-Infected Cervical Cancer Cells. *Molecules (Basel, Switzerland)* 20(7), 11830–11860.
- Pinzi, L. and Rastelli, G. (2019). Molecular Docking: Shifting Paradigms in Drug Discovery. *International journal of molecular sciences*, 20(18):4331.
- Pommier, Y. (2013). Drugging Topoisomerases: Lessons and Challenges. *ACS Chemical Biology*, 8:82–95.
- Prasasty, V.D., Eveline, I., & Noya, E.J. (2017). Potential Natural Inhibitor Bioactivities against High-Risk HPV-16, HPV-18 and HPV-52 of E6 Oncoprotein through Molecular Docking. *International journal of scientific research in science, engineering and technology*, 3, 39-45.
- Rodriguez-Mateos, A., Vauzour, D., Krueger, C.G. *et al.* Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Arch Toxicol* 88, 1803–1853 (2014).
- Schoeffler, A.J. and Berger, J.M. (2008). DNA topoisomerases: Harnessing and constraining energy to govern chromosome topology. *Quarterly Reviews of Biophysics*, 41:41–101.
- Singh, J., Petter, R.C., Baillie, T.A. and Whitty, A. (2011). The resurgence of covalent drugs. *Nature Reviews Drug Discovery*, 10:307–317.
- Stefaniu, A. (2019). Introductory Chapter: Molecular Docking and Molecular Dynamics Techniques to Achieve Rational Drug Design, Molecular Docking and Molecular Dynamics, Amalia Stefaniu, IntechOpen, DOI: 10.5772/intechopen.84200.
- Trott, O. and Olson, A.J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *Journal of Computational Chemistry*, 31:455-461
- Zaman, M. S., Chauhan, N., Yallapu, M. M., Gara, R. K., Maher, D. M., Kumari, S., Sikander, M., Khan, S., Zafar, N., Jaggi, M., & Chauhan, S. C. (2016). Curcumin Nanoformulation for Cervical Cancer Treatment. *Scientific reports*, 6, 20051. <https://doi.org/10.1038/srep20051>
- Zhang, Z., Cheng, B. and Tse-Dinh, Y. (2017). Crystal structure of a covalent intermediate in DNA cleavage and rejoining by Escherichia coli DNA topoisomerase I. *Proceedings of the National Academy of Sciences of the United States of America*, 108: 6939–6944.