ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Assessment of Lycopene Effects on Herpes Simplex - 1 in Tissue Culture.

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ABSTRACT

This study was planned to evaluate the antiviral effect of lycopene against HSV-1 infection compared with acyclovir. The protocol of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done for the determination of cell viability and antiviral effect of lycopene as compared with acyclovir by measuring drug half-maximal Effective concentration (EC_{50}) causing 50% inhibition in viral– induced cytopathic effects with different concentrations (5 µg/ml, 10µg/ml, 15µg/ml,20 µg/ml and 25µg/ml) of lycopene and acyclovir. The assay of in vitro studying of antiviral effect by MTT revealed a significant antiviral activity of lycopene (P= 0.015) against HSV-1 at a concentration of 25µg/ml. The peak of optimum acyclovir concentration was equal to 15 µg/ml (P=0.013). The half maximal effective of acyclovir concentration (EC_{50}) was 1.916 µg/ml, while lycopene EC_{50} was equal to 22.86 µg/ml. This study revealed a most important conclusion that Lycopene revealed a significant antiviral effect and anticytotoxic activity against HSV-1 infection in vitro.

Keywords: RD cell lines, cell culture, herpes simplex1, MTT assay, Lycopene, Acyclovir.

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ISSN: 0975-8585

INTRODUCTION

Herpes simplex-1 is (HSV1) widespread, enveloped and double stranded DNA agents which cause various infections in human. The virus causes recurrent infections of the nervous system located around the lips, eyes, mucous membrane of the oral cavity and genital [1]. The drugs found to be clinically useful in the treatment of HSV-1 are synthetic nucleosides such as acyclovir (ACV). The severe side effects and the emergence of drug-resistance mutants during long-term medication with these drugs have often limited their administration to patients [2,3]. Thus, the development of novel antiviral agents against this virus is still an important area of research. The majority of clinical HSV-1 isolates contain ACV resistant ($ACV^{R)}$ viruses, and the frequency of these viruses determines the isolate's ACV susceptibility phenotype: ACV^{R} or ACV-sensitive (ACV^{S}) [4,5,6]. Recently, Santoyo *et al.*,2014 turned the attention toward plant extracts that targeted viral nucleic acid as alternative antiviral drugs against HSV-1[7].

Plants are known as important sources of new chemical entities suitable for antiviral drug discovery and development [8]. Many investigators have reported the inhibitory effects of algal extracts on the replication of herpes simplex viruses [9]. Another study indicated that crude hot glycerin extract of fresh leaf *A. marina can* show antiviral activity against HSV-1 in vitro, which could lead to more studies for developing antiviral drugs against HSV-1 [10]. In silico model of antiviral drug assessment have been introduced. Recently, A prior in silico assessment of α-pinene against different rotavirus protein NSP1-6 and VP1-8 through the process of drug docking by in silico model stimulation program was carried out to predict a considerable antiviral effect for conducting in vitro and in vivo assessment [11]. The role of lycopene as antiviral substance is limited, thus we think this study is considered from the first trial to study the hypothesis of the role of lycopene as antiviral agent against HSV-1. The aim of the study was the studying of antiviral effect of lycopene compared with acyclovir against HSV-1 by MTT assay.

MATERIAL AND METHODS

In silico model of HSV-1 genes interactions with lycopene

A computerized simulation of test agent and target is a well-known approach in drug assessment through the process of docking. The structure of the ligand molecule Lycopene [PubChem: Compound ID: 10918539] retrieved from NCBI-PubChem Compound database [NCBI-PubChem Compound database http://pubchem.ncbi.nlm.nih.gov/].

Antiviral assay of lycopene and acyclovir

The antiviral activity of lycopene and Acyclovir against HSV-1 was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MTT colorimetric assay is an established method of determining cell viability in proliferation , cytotoxicity and antiviral drugs activity studies . This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes (in cultured cells), and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during MTT exposure. Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of viable cells in culture [12]. Cell lines were seeded onto 96 well plates with a concentration of 1.0 x $l0^5$ cells/ml. After incubation at 37°C for 24 to 48 hrs and when the confluent monolayer of RD cells was complete (80% - 100%), the virus ($TCID_{50}$= 1.0 x 10^5) was added into all wells except cells control wells then incubated for 1h. Different concentrations (5,10,15,20,25 µg/ml) of microtitered lycopene or Acyclovir were added to cultured wells at a final volume of 100 µl in each well except cells control and virus control wells in triplicate . The maximum concentration of DMSO (0.1%) was used as negative control and added on culture media regarded to cell and virus control cultivation.

After 48 hrs incubation at 37°C in 5% CO2, the microtiter 96 wells plates were marched out and transferred to biohazard safety cabinet by sterilized environments to avoid any contamination. All used wells media were discarded. The RD monolayers were washed by PBS solution to remove any residual amount of drugs (lycopene or acyclovir) that may be interacts with MTT reagents, since lycopene is a colored substance that may interact with absorption in reading step . Then 100 µl of maintenance media was added to all wells containing drugs treated cells, drugs untreated cells and blank wells. Then, MTT reagent (20 µl) was added to

ISSN: 0975-8585

each well. After 4 hrs of incubation at 37°C, 5% CO2 , the formazan particles were be formed as a mitochondrial enzymatic process of the non effected viable RD cells. The dead or viral effected cells did not form formazan particles because its mitochondria organelles were disrupted. The formazan was solubilized by adding diluted dimethylsulfoxide DMSO (1:1) in isopropanol on each wells including blank wells. The absorbance was read at 490 *nm* with a reference wavelength of *630 nm* by an ELISA reader. This protocol of MTT assay measurement was mentioned by many reports [13,14 ,15]. Mean blank absorption was subtracted from other samples and controls wells absorptions. Data were calculated as the inhibition percentage of viral growth and replication on RD cells. Viral inhibition rate was calculated as: [(Atv-Acv)/ (Acd-Acv)]x100. Atv indicates the absorbance of the lycopene or acyclovir with virus-infected cells while Acv indicates the absorbance of wells that contain cells and virus without drugs treatment. Acd represents the absorbance of the cell control in which is not treated with lycopene nor acyclovir [16]. The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the effective concentration which achieved 50% inhibition of virus-induced cytopathic effects [17].

Statistical analysis

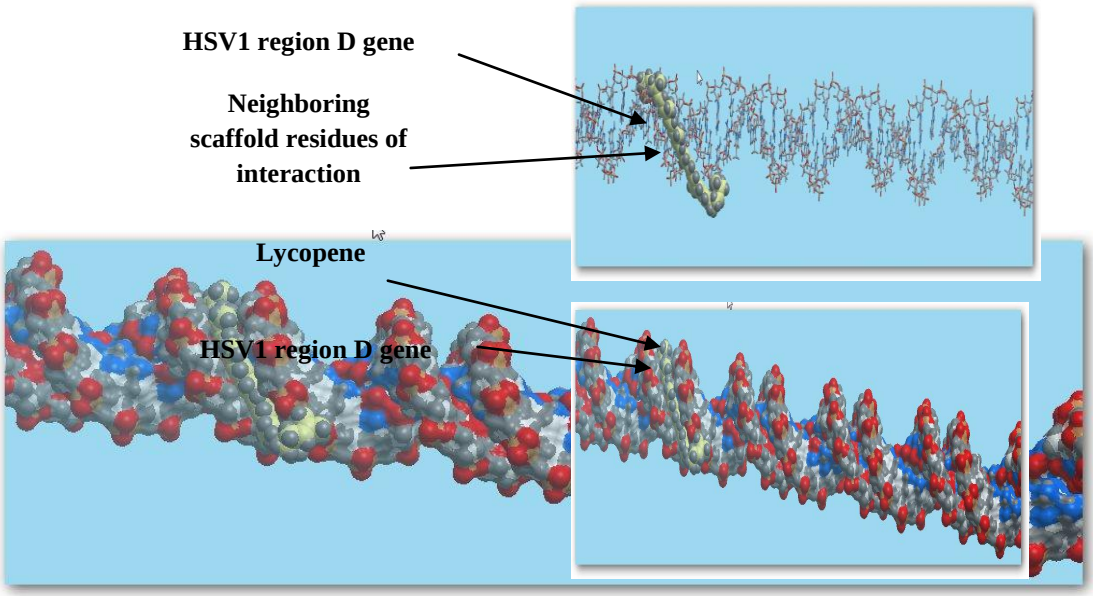
Statistical analyses were performed using SPSS 18.0 for windows. Inc. Data were expressed as Mean ± SD , two-tailed T test unless otherwise stated by ANOVA test. In all tests, $P<0.05$ was considered statistically significant. RMSD was used in the calculation of Insilico results of binding accuracy .R^2 was calculated by Pearson Correlation Coefficient. The half inhibitory concentration (EC_{50}) of acyclovir and lycopene was calculated by GraphPad Prism6.

RESULTS

In silico model of herpes virus 1 region D genome interactions with Lycopene

Results of the computerized model of HSV1 region D genome docking with Lycopene showed obvious Van der Vaals interaction of Lycopene force field with a scaffold like of nucleic acid residues. Another consideration was that lycopene showed obvious drug likeliness , residues likeliness and had a scaffold likeliness mode of inhibition of gene replication blocking (according to In silico model log file binding results), as in Project HS1Lycop, as demonstrated in whole protocol of docking **(Figure4-1)**.

Figure (4-1): In Silico results: ligand interacts with scaffold residues of region D of HSV1 genome with lycopene, this interaction occurred at minor groove by Wan der Waals force.



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Evaluation of Lycopene Antiviral activity

To evaluate any toxic effect of lycopene and acyclovir against RD cells, a concentration of 25µg/ml of lycopene/acyclovir was used. Firstly, cultured RD cells were treated with 25µg/ml of drugs. After 48hrs MTT assay was applied for the assessment of any toxic effect on cell viability. Lycopene did not exhibit any toxic effect on RD cells at a concentration of 25µg/ml as compared with non treated cells. Similar results of acyclovir were seen. This result gives an indication that CC_{50} of lycopene is more than 25 µg/mL. Thus anti-HSV1 activity was determined at concentrations lower than 25µg/mL of the lycopene and standard drug. This pre step was done for the optimization of lycopene concentration to be used in the subsequent processing via MTT assay for the determination of the antiviral effect and assessment of the drug EC_{50}. Therefore, all subsequent studies were done for lycopene and acyclovir at concentrations ≤ 25 µg/ml .

This study revealed high significant differences according to the viability of virally infected cells compared with non infected normal cells (P=0.008). The assessment of the viability was depending on the condensation of insoluble formazan particles as shown on **figure 4-5** (**A**&**B).** Results were also showing significant differences according to the virally infected without treatment as compared with lycopene treated groups (P= 0.015), with peak of lycopene antiviral effect at a concentration of 25 µg/ml. These results were viewed in **(Figure 4-2).** They show different groups involved virally infected without Lycopene treatment compared with those treated with 5,10,15, 20 and 25 µg/ml of Lycopene. Similar results of acyclovir show that graduated Lycopene treatment result in subsequent elevation of which indicate to the exceeding of cell viability (**Figure 4-2).** HSV-1 infected RD cells that were treated with 5 µg/ml of lycopene showed inhibition percentage of about 2.06%, but this elevation was statistically not significant (P=0.922). This activity was elevated subsequently with 5 fold treatment; therefore, lycopene exhibits protective activity against the virus. Our results shown in **(Table 4-1)** revealed that lycopene affects HSV-1 growth and with expression to its protective activity on RD cells by inhibition of 22.31 % of virus growth at 10 µg/ml concentration. This inhibition percentage elevated gradually with subsequently 5 fold increasing in lycopene concentration to reach 44.62 %, 57.85 % in inhibition percentage at 15 µg/ml and 20 µg/ml respectively**.**

The higher antiviral activity of lycopene was shown at higher concentration (25µg/ml) with 80.16 % inhibition in virus growth (results were statistically significant(P=0.015). The increasing in live RD resulting from lycopene rule in limitation of viral infection effects treated RD cells as compared with those untreated **(Table-4-1)** and this Shows inhibitory effect of lycopene against viral infections in dose-dependent manor. From the data obtained from MTT assay for antiviral action of lycopene, the dose that inhibited viral infection by 50% (EC_{50}), the effective concentration required to inhibit 50% virus infection compared to infected cells without treatment, was determined from the curve of plotting the inhibition of the virus yield versus the concentration of the samples by GravPad Prism 6, EC_{50} of lycopene was 22.86 µg/ml.

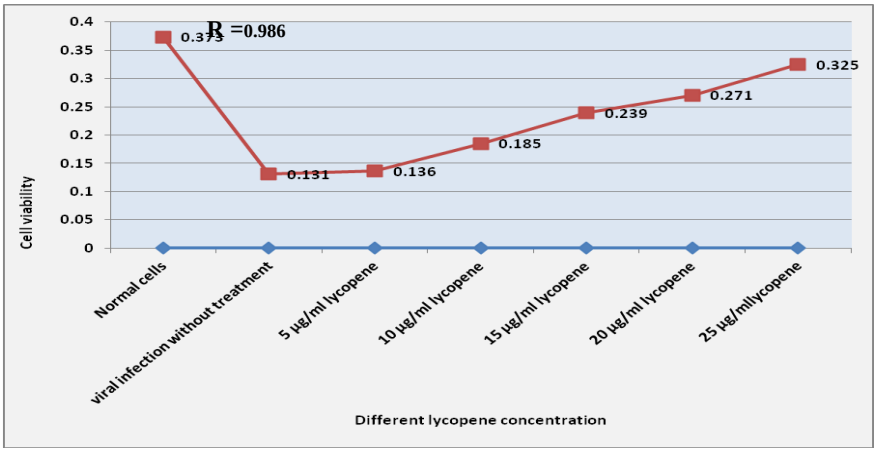


Figure (4-2): Results of MTT assay presented by Mean ±SD of OD,after 48 hrs of HSV1 infection on RD cell line. This figure showing different groups involved virally infected without lycopene treatment as compared with those treated with 5,10,15,20 and 25 µg/ml of lycopene.

ISSN: 0975-8585

Table (4-1): Antiviral effect (inhibition% of HSV-1 effects) of lycopene presented by Mean ±SD of OD at 490nm, after 48 hrs of HSV1 infection on RD cell line, and MTT test.

Groups	No.	OD * Mean ±SD	Inhibition%of HSV-1 effects on the RD cells	*P-value (Two-tailed T test)
Normal cells	3wells	0.373±0.045	Control	0.008
Viral infection only (0 µg/ml of lycopene)	3 wells	0.131±0.072	Control2	**0.008
lycopene 5µg/ml	3 wells	0.136±0.042	2.06 %	0.922
lycopene 10 µg/ml	3 wells	0.185±0.083	22.31 %	0.444
lycopene 15 µg/ml	3 wells	0.239±0.055	44.62 %	0.109
lycopene 20 µg/ml	3 wells	0.271±0.044	57.85 %	0.046
lycopene 25 µg/ml	3 wells	0.325±0.039	80.16 %	0.015

*** If P value ≤ 0.05, then the test is regarded as significant;** One way Anova test; R = 0.9860**

Evaluation of Acyclovir Antiviral activity

The results of antiviral activity of acyclovir against HSV-1 also revealed high significant differences (P=0.008),**(Table 4-2)** with peak of viral infection inhibition at 15 µg/ml of acyclovir**.** It is very obvious that graduated acyclovir treatment results in subsequent elevation of OD that referred to the exceeding of cell viability **(Figure 4-3).** Protection effect of Acyclovir was obvious with graduated treatment**.** The halve-maximal effective concentration(EC_{50}) was calculated by GraphPad Prism 6 to show acyclovir concentration causing of 50% formazan particles formation in infected cells as forming as in control cells (EC_{50}) was (1.916 µg/ml).These results gave an indication about the optimum concentration that regarded suitable as antiviral and not toxic to the cells, as shown in **(Table 4-2) and Figure (4-4).**

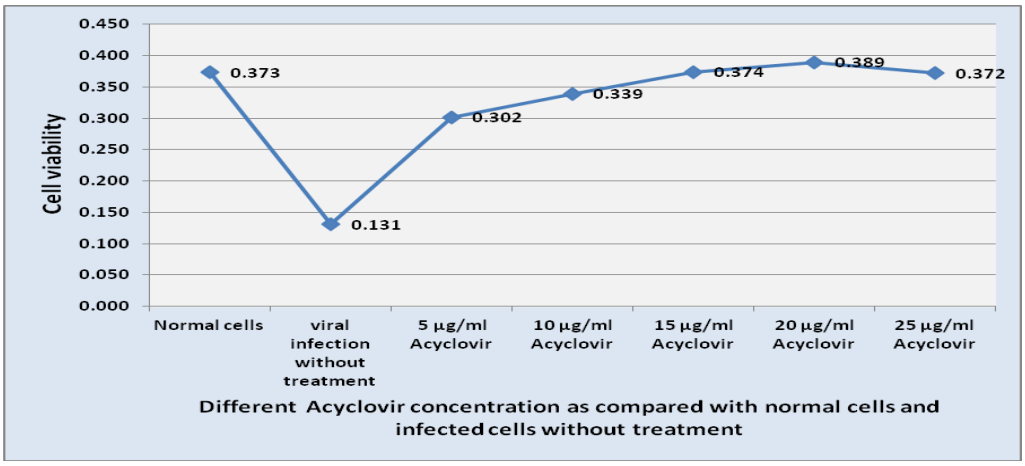
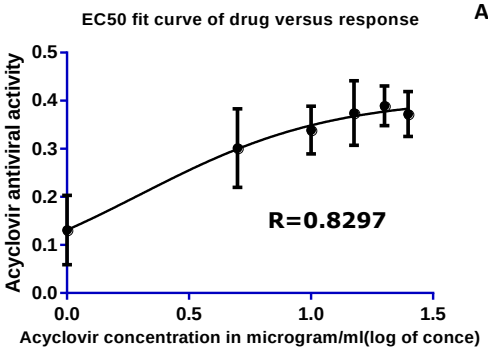
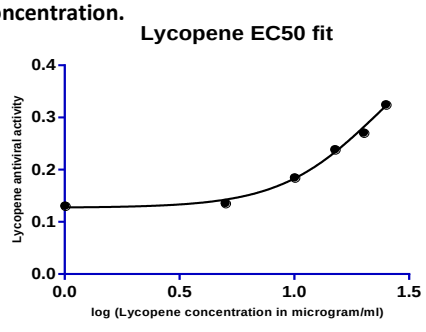


Figure (4-3): Results of MTT assay presented by Mean ±SD of OD,after 48 hrs of HSV1 infection on RD cell line. This figure showing different groups involved virally infected without Acyclovir treatment as compared with those treated with 5,10,15,20 and 25 µg/ml of Acyclovir

Figure (4-4): EC50 fit of Acyclovir after 48 hrs of HSV1 infection on RD cell line. A-This Activity versus log of concentration; B-Activity versus concentration.





ISSN: 0975-8585

Table (4-2): Antiviral effect (inhibition of HSV-1 effects) of acyclovir presented by Mean ±SD of ODat 490nm, after 48 hrs of HSV1 infection on RD cell line, and MTT test.

Groups	No.	OD Mean ±SD	Inhibition % of HSV-1 effects on the RD cells	*P-value (Two-tailed T test)
Normal cells	3wells	0.373±0.045	Control 1	*0.008
Viral infection only (0 µg/ml of acyclovir)	3 wells	0.131±0.072	Control2	**0.002
acyclovir 5 µg/ml	3 wells	0.302±0.082	70.66 %	0.053
acyclovir 10 µg/ml	3 wells	0.339±0.050	85.95 %	0.015
acyclovir 15 µg/ml	3 wells	0.374±0.067	100 %	0.013
acyclovir 20 µg/ml	3 wells	0.389±0.041	100%	0.006
acyclovir 25 µg/ml	3 wells	0.372±0.047	99.50 %	0.008

*** If P value ≤ 0.05, then the test is regarded as significant;** One way Anova test**

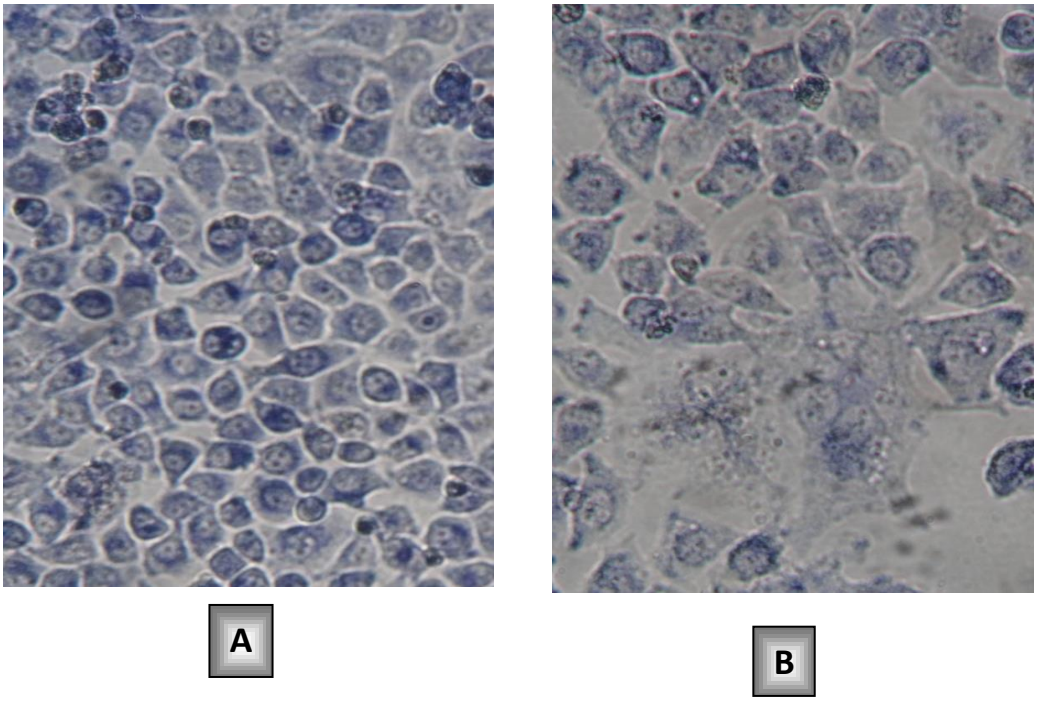


Figure 4-5 : Insoluble formazan particles formation as shown by MTT assay: A- Normal RD cells ; B- HSV-1 infected RD cells.

DISCUSSION

As demonstrated in whole protocol of docking **(Figure4-1)**. Lycopene showed promising parameters in therapeutic approach HSV1. It showed in silico parameters as antiviral agent by Wan der Waals interaction of Lycopene force field with a scaffold like minor groove at twisted region D gene of HSV-1.

This interaction between lycopene and this gene were referred to as reliable mechanism of impairment of herpesvirus replication and halting of the action of polymerases enzymes (according to In silico model software reliability). Our results made lycopene a rational candidate for ongoing in vitro assessments on herpesvirus models. Lycopene showed a considerable interaction with other viral genetic regions (as scanned by In silico model). However, model of HSV 1 genome-lycopene was based on the rational of most important virulence factor in herpesvirus (U_L and U_S regions) and due to the structural characteristic of lycopene. In our study, we hypothesized that lycopene has an antiviral activity. In another study that achieved

in open randomized clinical trials with a tomato-based field to develop a food for special medical purposes (FSMP), and in a scenario of using FSMP as adjuvant treatment to the pharmacological therapy with pegilated interferon and ribavirin, to assess its efficacy as adjuvant therapy in patients with chronic hepatitis C virus (HCV), it seemed that FSMP was effective in improving carotenoid status in healthy subjects [18]. However, little is known about the rule of lycopene as antiviral agent and hence, our study can be regarded from first studies that pave the way for the use of lycopene as antiviral, and give future as alternative antiherpetic therapeutic agent.

The present study is an attempt to elucidate the action mechanism of naturally occurring lycopene in inhibiting viral DNA replication process, thus providing an evidence for its development as a novel anti-herpetic drug. We have performed docking and molecular dynamics simulation studies to elucidate the binding mechanism of lycopene onto the structure of region D gene of Herpes simplex virus1. Our docking simulations results give a high binding affinity of the ligand to the receptor. The value of R^2, the coefficient of determination (Pearson Correlation Coefficient) was 0.5069, and this was regarded as positive correlation. Simulations supported the hypothesis that lycopene is a potential ligand to target/inhibit DNA replication of herpes simplex virus-1. Results of in silico study will also guide the design of selective inhibitors of DNA with high specificity and potent activity in order to strengthen the therapeutic arsenal available today against the dangerous biological warfare agent represented by Herpes Simplex Virus1.

The results of MTT assay suggest that lycopene has association with antiviral activity and protective rule against HSV-1 infections, where reduced the cytopathic effects are related to HSV1 infection on RD cells (in vitro) for about half when cells were treated with 22.86 µg/ml of lycopene, another study showed that lycopene has a significant antiviral activity and viral growth limitation (P= 0.013) [19]**.** This antiviral activity may be attributed to behavior of lycopene as gen blocker. Insilico model, results gave high binding affinity of lycopene ligand with receptors of gD gene site on HSV1 viral genome result in obstruction through gD protein expression process.

The results may also came in agreement with the finding that acyclovir EC_{50} against herpes simplex virus isolates ranges from 0.02 to 13.5 µg/ml for HSV-1 [20, 21]. The inhibitory activity of acyclovir is highly selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV1. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleotide analogue. The monophosphate is further converted into diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. *In vitro*, acyclovir triphosphate stops replication of herpes viral DNA. This is accomplished in three ways: firstly, competitive inhibition of viral DNA polymerase; Secondly, incorporation into and termination of the growing viral DNA chain; and finally inactivation of the viral DNA polymerase. Aciclovir by the parenteral route is the drug of choice in severe, acute HSV infections. The acyclic guanosine derivate aciclovir is specifically activated by the viral enzyme thymidine kinase of HSV to its monophosphate. The greater antiviral activity of acyclovir against HSV is due to its more efficient phosphorylation by the viral TK [22]. We concluded that lycopene has strong antiviral activity against HSV-1 infection also with low cytotoxicity effects relatively.

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