



Assessment Of Pomegranate Peel And Seeds Extracts Antiviral Potential And Antioxidant Activity Against HAV And HSV-1 Virus Models And Related In Silico Computational Study

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Abstract

The antiviral potential of pomegranate ethanolic extracts was monitored against herpes simplex virus type 1 (HSV-1) and hepatitis A virus (HAV). GCMS was performed to monitor the bioactive containment. Cell viability and antiviral activity of these extracts were assessed using endpoint assay, plaque reduction, and virucidal approaches against the target viruses. The IC50 of seed extract was about 31.216 mg/ml. HAV and HSV-1 infectivity titers were reduced by 23.50% and 25.%, respectively. While PSE reduced HAV and HSV-1 by 11.66% and 5.55%, respectively, using the direct method and 41.17% and 25% using the indirect method, PSE reduced HAV and HSV-1 titers by 29.33% and 11.17%, respectively. Upon inspection, it was noted that the myxovirus resistance protein (MX-A gene) expression levels were significantly elevated in PPE/PSE treated cells compared with negative control. Moreover, the concentration of oxidative stress biomarkers, including Glutathione Reductase (GSH), Reactive Oxygen Species (ROS), Malondialdehyde (MDA), and Nitric Oxide (NO), were assessed in PPE and PSE-treated Vero cells compared to negative control cells. The results indicated that pomegranate peel could be a promising herbal medicine ingredient targeting HAV and HSV-1, showing greater efficacy than pomegranate seeds. The integration of bioactive products was considered using molecular docking analyses.

Keywords: Pomegranate ;HAV; HSV-1; GC-MASS; Antiviral; IC50; MX-gene; oxidative stress; Docking study.

1. Introduction

Pathogenic viruses are among the most common causes of significant morbidity and mortality rates in humans. Viral replication requires cell metabolism and cellular machinery [1]. According to the World Health Organization (WHO), Herpes simplex virus type 1 (HSV-1) is primarily transmitted through oral-to-oral contact to cause oral herpes infection, which can result in painful sores in or around the mouth ("cold sores"). In 2016, an estimated 3.7 billion people were infected with HSV-1, accounting for approximately 67% of the world's population aged from 0 to 49. The majority of these infections were oral; however, genital HSV-1 infection was estimated to affect between 122 million and 192 million people. Herpes simplex virus type 1 is one of the most prevalent Herpesviridae viruses [2].

The new WHO Global Health Sector Strategy (GHSS) on viral hepatitis, 2022-2030, which was recently reviewed and noted at the World Health Assembly, will play an important role in this summit. The strategy contains operational and strategic shifts to ensure that we are on track to meet the 2030 goal of ending viral hepatitis. In general, it is assumed to be the inhibition of viral attachment to host cells by causing damage to viral capsids or changing receptors on cell membranes [3].

Also, interferons (IFNs) can impede virus replication by inducing the production of IFN-stimulated genes (ISGs), such as the myxo-virus resistance (Mx) protein [4]. It also demonstrated that MX proteins had antiviral properties in numerous species. Mx research is still active today, providing insights into the never-ending battle between viruses and their hosts [5]. Creating a vaccination against viruses is difficult due to genetic variety and hypermutation [6]. So, there is a greater chance of discovering new antiviral medications that target viral proteins or host factors [7]. The various benefits of natural plant antimicrobials have sparked greater study and demand for such alternatives.

The *Punica granatum* plant, known as the pomegranate, is part of the Lythraceae family and is native to central and western Asia. It grows to a height of 6 to 20 feet (rarely to 30 feet) [8]. Pomegranate (*Punica granatum* L.) fruits are high in

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polyphenols such as ellagitannins (ETs), gallic acid (GA), ellagic acid (EA) and its glycosylated derivatives, and anthocyanins [9]. *Punica granatum* L (pomegranate) is a medicinal plant that has long been used to heal ulcers, diarrhea, and male infertility. It contains various pharmacological properties, including anti-diabetic, anti-tumor, anti-inflammatory, anti-malaria, anti-fibrotic, anti-fungal, anti-bacterial, and other benefits. Pomegranate also acts as an antiviral [10].

The peel of pomegranate fruit is typically discarded as waste. Still, chemical analysis of peel extracts revealed that it is also a rich source of bioactive polyphenols [11]. There is also no evidence of toxic effects on body organs such as the heart, liver, or kidney [12]. So, we worked on the hypothesis that pomegranate peel and seed extracts may be useful in preventing the spread of viruses. Plaque assay is an essential approach for measuring more accurate infectious particles of contagious viruses [13]. Pomegranate can effectively improve some oxidative factors and thus has antioxidant activity due to its high content of phenolic components [14]. The term "oxidative stress" refers to an imbalance between the creation of reactive oxygen/nitrogen species (ROS/RNS) and the cell's ability to neutralize them by anti-oxidative defense mechanisms [15]. Oxidative stress parameters such as GSH, ROS, MDA, and NO. Computational docking can be used to predict bound conformations and free energies of binding for small molecule ligands to macromolecular targets. Docking is frequently utilized in the study of biomolecular interactions and processes, as well as in structure-based medication discovery [16]. The docking research looked at the binding manner and interaction of pomegranate extract chemicals with certain viral enzymes. Some chemical compounds interact with viral enzymes.

2. Materials and methods

Pomegranate plant

About 5 Kg of Pomegranate were purchased from the local market, with seeds separated from the peel. Both peel and seeds were air dried and ground into fine powder.

Pomegranate peel and seeds extraction

Plant materials were dried at 40°C in a hot air oven. Dried plants were ground into a fine powder, 250 gm Powdered plants were homogenized, then macerated in a stoppered container with 2.5 liters of 80% ethanol and allowed to stand at room temperature for 3 days. For conventional extraction, the extract and powder were placed in a sonicator at 40 °C for 60 min. The extracts were filtered & concentrated under vacuum at 40 °C using Rota vapor to provide crude extract(gm). They reconstituted the Crude extract in 10 ml ethanol [17].

GC-MS analysis

GC-MS subjected pomegranate peel and seed extracts to qualitative and quantitative analysis.

Viral models

Two viruses were used as examples for RNA and DNA viruses, namely Herpes simplex virus type 1(HSV-1) and Hepatitis A virus (HAV), which were kindly supplied by the International Centre for Advanced Researches (ICTAR -Egypt).

Cellline

African green monkey kidney-derived Vero cells (Vero, CCL-81) were kindly supplied from the International Centre for Advanced Researches (ICTAR -Egypt).

Interferon- α -2a(positive antiviral drug)

Interferon- α 2a was kindly supplied by the national authority for control of vaccine and biological product NORTH-B,Egypt.

Cell Viability (MTT Assay)

Vero cells were grown in 75 cm² surface area cell culture flasks (SPL, Korea) as previously described [18], where confluent sheets were treated for 5 minutes using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylene diamine tetra acetic acid (GIBCO-USA). Cells were suspended in a growth medium and plated in 96-well cell culture plates at a density of 2 x 10⁵ cells/ml and incubated at 37°C for 24 hours to achieve confluency. The growth medium was removed carefully, and cells were washed with fresh medium containing 2 fold serially diluted extract (4 wells/dilution). After 24 hours, dead cells were washed out with PBS, and 50 μ l of MTT stock solution (0.5 mg/ml) was added to each well. After 4 hours of incubation, the supernatant was discarded, and the formazan precipitates were solubilized with 50 μ l of dimethyl sulfoxide per well (DMSO, Sigma Aldrich, USA). Plates were incubated in the dark for 30 minutes at 37°C, and absorbance was measured at 570 nm using a microplate reader (ELX -800, Biotek- USA). The percentage of cell viability was estimated using the following formula:

Cell viability percentage = (OD of treated cells / OD of untreated cells) X 100.

Master Plex 2010 Software version 22.00.77 was used to calculate the IC₅₀ values.

Viruses infectivity titre

The infectivity titer of test Viruses (HAV&HSV-1) stocks were measured according to Bussereau, *et al.* [18], as virus seeds were 10-fold serially diluted in MEME supplemented with 2% FCS. Virus dilutions were dispensed to pre-

cultured Vero cells at 0.1 mL/well. Plates were incubated at 37°C and inspected for 7 days post-virus inoculation using an inverted microscope (Hund-Germany) to identify cytopathic effect (CPE). The 50% endpoint was calculated using the Muench equation [19].

Antivirus mechanism

a-Indirect antivirus activity (pre-infection)

Vero cells were treated with a safe concentration of test extracts for 24 hours. The treatment media was decanted. Test viruses were 10-fold serially diluted and then dispensed to the pre-cultured Vero cells. The virus infectivity titer was determined according to Fenard, et al. [20], and the antiviral activity was calculated by subtracting the virus infectivity titer of treated cells from that in untreated cells.

b-Direct antivirus activity (post-infection)

Vero cells were pre-treated with the test virus dilutions for 1 h, as previously. Unbound virus particles were washed out using phosphate buffer saline (PBS), and the safe concentration of test extracts was dispensed to infected wells. Plates were incubated and examined daily for detection of cytopathic effect (CPE). The endpoint was determined as previous, and the reduction % of infectivity titers as well.

c-Virucidal activity

Test virus models were added to the safe concentration of both seed and peel extracts as 1/10. Mix tubes were incubated at 37°C, and a sample of 1 ml was collected at 1,2,4,6,8,12,24 hrs. Test extracts -viruses mixed at time intervals were processed as previously to prepare 10-fold serial dilutions. Virus model dilutions were dispensed to pre-cultured Vero cells post-decanting of growth medium. Plates were incubated for 7 days, and microscopic examination was performed daily to detect CPE. The endpoint of the treated virus was calculated as previously the infectivity titer relative to time was calculated.

d-plaque reduction assay

Vero cells were cultured in MEM-E and incubated at 37°C in a 5% CO₂ incubator (Hera-cell, USA). Cells were seeded as 5 x 10⁵ cells /mL in a 6-well plate (3 ml/well) 24 h before the performance of plaque reduction assay (PRA). Tenfold serial dilutions of test viruses were dispensed to pre-cultured Vero cells for 1 h. The inoculum containing the unbound virus particles was decanted. A safe concentration of test extract in 2x MEM-E medium was mixed to an equal volume of 2% agarose, and the mix was kept at 45°C to keep agarose liquified and added to the infected wells (2ml/well) in triplicates/plates were left at room temperature to solidify and incubated at 37°C in and inverted positions. Plates were examined 3 days post-processing. Plates showed CPE were fixed with 5-10 % formal saline for 1 h. Floating agarose was discarded, and plates were washed and stained with 1% crystal violet. Plaques in treated and untreated wells were counted. The difference refers to the reduction in %.

Detection of Mx-Agene by Real-time PCR

Total RNA was isolated from control untreated, PPE, and PSE-treated Vero cells according to the manufacturer's procedure using the previously Mx Gene JET RNA Purification kit (Fermentus-UK). The samples were stored at -80°C till use, and the Mx gene expression levels were determined using the primers F 5'-AAA TGG CTC AAG AGG TGGA-3' R 5'-TAT CGC TGA CAG TTTG GGTG-3'. Vero cells Mx gene expression levels were measured using the same real-time PCR mixture, amplification settings, and No of cycle count as previously described [21]. Melting curves and standard curves were tested.

Biochemistry

Glutathione reductase (GSH) detection

Detection of GSH was performed according to Yiiksel, et al. [22] based on the reduction of 5,5' di-thiobis (2 - nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration, and its absorbance can be measured at 405 nm.

Reactive Oxygen species (ROS)

ROS ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-ROS antibody and ROS-HRP conjugate. The assay samples and buffer were incubated together with ROS-HRP conjugate in a pre-coated plate for one hour. After the incubation period, the previous sample and conjugate contents were decanted, and the plate was washed five times. The wells were then incubated with a substrate for the HRP enzyme. The product of the enzyme-substrate reaction forms a blue-colored complex. Finally, a stop solution was added, which turned yellow. The intensity of color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the ROS concentration [23].

Malondialdehyde (MDA) detection

MDA detected by Thio-barbituric acid (TBA) reacts with malondialdehyde (MDA) in the acidic medium at a temperature of 95°C for 30 min to form a Thio-barbituric acid reactive product. The absorbance of the resultant pink product can be measured at 534 nm [24].

Nitric oxide (NO) estimation

Nitric oxide (NO) was estimated by the colorimetric method of Vandenberg, et al. [25]; in the acidic medium and in the presence of nitrite, the formed nitrous acid diazotize sulphanilamide, and the product is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color, which could be measured at 540 nm.

Docking Studies

Docking studies were carried out utilizing Discovery Studio 4.0. All bound waters, ligands, and cofactors were removed from the protein before the docking process. The 3D crystal structure of the targeted macro-molecules was obtained from the protein data bank (PDB ID: 4M5W, 3B25, 2KI5, 3NF7 and 3ASZ). 3D structures were constructed using Chem 3D ultra 12.0 software [Molecular Modeling and Analysis, Cambridge Soft Corporation, USA (2010)]. The selected compounds were energetically minimized using MOPAC (semi-empirical quantum mechanics), international coordinate, and saved as MDL MolFile (*.mol).

Statistical Analysis

All statistical calculations were done using Microsoft Excel version 16 and SPSS (statistic package for the social science version 27.00) statistical program at 0.05, 0.01, and 0.001 level of probability (Snedecor and Cochran, 1982). Results of the experiments were subjected to one-way ANOVA with the least significant difference (LSD) and Tukey's test was presented using mean \pm standard deviation. The figures are drawn with Prism 8.01 (GraphPad Software, San Diego, CA, USA). Snedecor, G.W., Cochran, W.G. 1973. Statistical Methods. 6th ed., Iowa State University Press, Iowa, USA. 593.

3. Result

GC-MS analysis

Pomegranate peel and seeds extracts were subjected to qualitative and quantitative analysis by GC-MS. The content compounds pomegranate seed and peel extracts were chemically identified, and the components MW formulae, the area under the curve (AUC), and retention time (RT) were tabulated as shown in (Table 1-2).

Table 1. Identification of seed extracted compounds using GC-MS

RT	Compound Name For Seed Extract products	Area%	Formula	MW
5.24	2,5-Dihydroxyacetophenone, bis(trimethylsilyl) ether	0.07	C ₁₄ H ₂₄ O ₃ Si ₂	296
5.87	Melibiose	2.21	C ₁₂ H ₂₂ O ₁₁	342
6.17	7,3',4',5'-Tetramethoxyflavanone	0.16	C ₁₉ H ₂₀ O ₆	344
6.38	6-OXABICYCLO[3.1.0]HEXAN-3-ONE	2.43	C ₅ H ₆ O ₂	98
6.55	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2.69	C ₆ H ₈ O ₄	144
7.19	INDOLIZINE	1.00	C ₈ H ₇ N	117
7.26	2-tert-Butyl-4-methyl-1,3-oxathiolan-5-one	1.38	C ₈ H ₁₄ O ₂ S	174
7.88	2-Deoxyribose diisopropylmercaptal	2.93	C ₁₁ H ₂₄ O ₃ S ₂	268
8.12	Diisopropylsilylether	0.33	C ₈ H ₂₀ O _{Si}	160
8.34	Zileuton	0.63	C ₁₁ H ₁₂ N ₂ O ₂ S	236
8.57	1-Oxacyclopentadecan-2-one, 15-isopropenyl	0.64	C ₁₇ H ₃₀ O ₂	266
8.64	1,2-Dimethyl-4-oxocyclohex-2-enecarboxaldehyde	1.23	C ₉ H ₁₂ O ₂	152

9.42	Desulphosinigrin	1.87	C ₁₀ H ₁₇ NO ₆ S	279
9.64	Melezitose	1.02	C ₁₈ H ₃₂ O ₁₆	504
10.37	Methyl2-methyl-3-oxobutylidithiocarbamate	0.46	C ₇ H ₁₃ NOS ₂	191
10.99	2-PROPYL-TETRAHYDRO-PYRAN-3-OL	1.74	C ₈ H ₁₆ O ₂	144
11.05	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	3.09	C ₆ H ₈ O ₄	144
11.47	Cyclohexanecarboxylicacid,2-hydroxy-,ethylester	4.44	C ₆ H ₁₂ N ₂ S	172
13.24	CYSTIN	0.19	C ₆ H ₁₂ N ₂ O ₄ S ₂	240
14.92	5-Hydroxymethylfurfural	30.73	C ₆ H ₆ O ₃	126
26.79	PENTADECANOICACID,14-METHYL-,METHYLESTER	2.34	C ₁₇ H ₃₄ O ₂	242
28.08	Palmiticacid,ethylester	0.56	C ₁₈ H ₃₆ O ₂	284
28.67	HEXADECANOICACID	6.49	C ₁₆ H ₃₂ O ₂	256
29.64	(9E)-8-Methyl-9-tetradecenylacetate	0.32	C ₁₇ H ₃₂ O ₂	268
29.98	7,10-Octadecadienoicacid,methylester	5.42	C ₁₉ H ₃₄ O ₂	294
30.1	10-Octadecenoicacid,methylester	8.85	C ₁₉ H ₃₆ O ₂	296
30.55	OCTADECANOICACID,METHYLESTER	1.23	C ₁₉ H ₃₈ O ₂	298
30.88	Dasycarpidan-1-methanol,acetate(ester)	0.25	C ₂₀ H ₂₆ N ₂ O ₂	326
31.74	9-OCTADECENOICACID(Z)-(OleicAcid)	10.44	C ₁₈ H ₃₄ O ₂	282
32.14	STEARATE	4.61	C ₁₈ H ₃₆ O ₂	284
32.68	SAFFLOWER OIL	0.14	C ₂₁ H ₂₂ O ₁₁	450
37.14	3',4',7-TRIMETHYLQUERCETIN	0.15	C ₁₈ H ₁₆ O ₇	344

Table 2. Identification of peel extracted compounds using GCMS.

RT	Compound Name for peel extract products	Area%	Formula	MW
5.04	ACETICACID,ETHOXY-,ETHYLESTER	0.44	C ₆ H ₁₂ O ₃	132
5.31	1-Methoxybicyclo[2,2,2]oct-5-en-2-ylmethylketone	0.75	C ₁₁ H ₁₆ O ₂	180
5.44	11,13-Dimethyl-12-tetradecen-1-olacetate	0.56	C ₁₈ H ₃₄ O ₂	282
5.55	3-Hydroxy-2,5,5,8a-tetramethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylicacid,methylester	0.19	C ₁₆ H ₂₆ O ₃	266
5.91	CYTIDINE,N-ACETYL	3.87	C ₁₁ H ₁₅ N ₃ O ₆	285
6.12	(2Z)-6,6-DIMETHOXY-3-METHYL-2-HEXENYLACETATE	0.09	C ₁₁ H ₂₀ O ₄	216
6.38	HEXADECANOICACID	2.63	C ₁₆ H ₃₂ O ₂	256
6.55	Ethyl2-hydroxycyclohexanecarboxylate	2.31	C ₉ H ₁₆ O ₃	172
7.18	2-TERT-BUTYL-4-METHYL-1,3-OXATHIOLAN-5-ONE	0.64	C ₈ H ₁₄ O ₂ S	174
7.25	2-tert-Butyl-4-(1-hydroxy-2-cyclohexen-1-yl)-4-methyl-1,3-oxathiolan-5-one	0.89	C ₁₄ H ₂₂ O ₃ S	270
7.54	2'-Hydroxy-2,5,6'-trimethoxychalcone	0.08	C ₁₈ H ₁₈ O ₅	314
7.64	2-METHYLBICYCLO[2.2.1]HEPT-2-YLACETATE	0.1	C ₁₀ H ₁₁ D ₃ O ₂	173
7.81	Dimethyl2-hydroxy-2-methylbutane-1,4-dioate	2.69	C ₇ H ₁₂ O ₅	176
7.91	Diisopropyl(ethoxy)silane	2.96	C ₈ H ₂₀ OSi	160
8.33	Zileuton	0.74	C ₁₁ H ₁₂ N ₂ O ₂ S	236
8.56	E-10-Methyl-11-tetradecen-1-olpropionate	1.02	C ₁₈ H ₃₄ O ₂	282
8.94	(14E)-14-OCTADECENAL	0.21	C ₁₈ H ₃₄ O	266
9.16	9-Octadecenamide,(Z)-	0.27	C ₁₈ H ₃₅ NO	281
9.39	6-O-Acetylhexopyranose	0.64	C ₈ H ₁₄ O ₇	222

9.63	Maltose	1.17	C ₁₂ H ₂₂ O ₁₁	342
10.39	(L)-ARGININE	0.35	C ₆ H ₁₄ N ₄ O ₂	174
10.95	1,3-Diazocane-2-thione	1.54	C ₆ H ₁₂ N ₂ S	144
11.21	Tryptamine	0.18	C ₁₀ H ₁₂ N ₂	160
11.59	L-Valine,N-[2-(chloroimino)-3-methyl-1-oxobutyl]-	0.74	C ₁₀ H ₁₇ ClN ₂ O ₃	248
11.85	4-Amino-1,5-pentandioicacid	0.64	C ₇ H ₁₃ NO ₄	175
14.9	5-Hydroxymethylfurfural	26.11	C ₆ H ₆ O ₃	126
23.93	17-Octadecynoicacid	0.42	C ₁₈ H ₃₂ O ₂	280
26.78	METHYL14-METHYLPENTADECANOATE	1.21	C ₁₇ H ₃₄ O ₂	270
28.07	Palmiticacid,ethylester	0.78	C ₁₈ H ₃₆ O ₂	284
28.62	n-Hexadecanoicacid	7.35	C ₁₆ H ₃₂ O ₂	254
29.37	Methyl10-octadecynoate	8.04	C ₁₉ H ₃₄ O ₂	294
29.95	Linolelaidicacid,methylester	3.87	C ₁₉ H ₃₄ O ₂	294
30.06	Oleicacid,methylester	4.64	C ₁₉ H ₃₆ O ₂	296
30.53	Methyl11-(3-pentyl-2-oxiranyl)undecanoate,cis-	1.16	C ₁₉ H ₃₆ O ₃	312
31.21	Ethyliso-allocholate	0.2	C ₂₆ H ₄₄ O ₅	436
31.71	trans-13-Octadecenoicacid	9.63	C ₁₈ H ₃₄ O ₂	282
32.11	STEARATE	6.25	C ₁₈ H ₃₆ O ₂	284
33.13	Z-(13,14-Epoxy)tetradec-11-en-1-olacetate	0.25	C ₁₆ H ₂₈ O ₃	268
37.45	Di-n-octylphthalate	4.5	C ₂₄ H ₃₈ O ₄	390

Area% in GC-MS analysis result refers to the high estamount compound of both extracts was 5-Hydroxymethylfurfuralasa majour compound which slightly larger in seeds extract, followed by oleic acid in seeds extract and trans1,3 octadecenoic acid in peel extract.

3.2. CYTOTOXICITY

The toxicity of both PPE and PSE was extract and concentration-dependent as Vero cells were less sensitive to peel extract than in the case of seeds,while viability was concentration-dependent as viability increased as long as the concentration decreased, recording a minimal inhibitory concentration IC₅₀ of 31.216 mg/ml in case of seeds extract the application and our study results refer to a high concentration of extracts, and peel extract improves cell viability and growth, the viability % was 55.78 while in case of seeds, the viability % was 21. Once a threshold is reached, the growth rate shows almost no difference. At the high concentration of extract, the inhibitory or toxic effect is observed in the case of seed extract more than in the case of peel extract. as shown in (Figure 1: A - B). The safe concentration was chosen at 6.25 mg/ml, which is the lowest concentration that gives a high viability % of 94.8% in the case of peel extract and 86.44% in seeds extract.

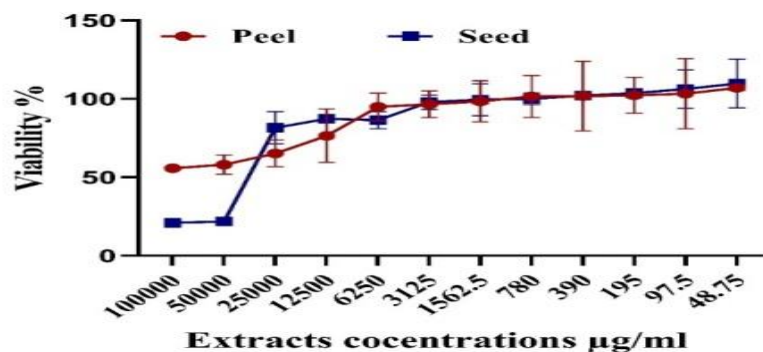


Fig.1- A.The viability percentage of concentration of the extracts. (Evaluation of cell viability of pomegranatepeeland seeds relative to different concentrations, using MTT assay).

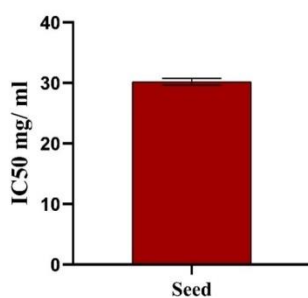


Fig.1-B.Assessment of IC₅₀ values of PPE & PSE post-Verocellines using MTT assay; IC₅₀ value of PPE not detected.

3.3.Antiviral activity of extracts against test viruses

The inhibitory effect of the ethanolic peel and seed spomegranate extracts were estimated against HSV-1 & HAV, and interferon was used as positive control. The overall results are illustrated in (tables 3,4,5,6), figures 2(A-B) and 3(A-B).

A-Indirect method antiviral evaluation

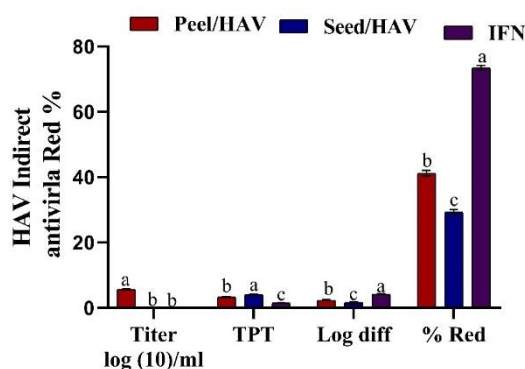


Fig. 2-A. Indirect method antiviral evaluation against Hepatitis A virus (HAV). %Red.: Reduction percent, TPT: Titre post-treatment. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

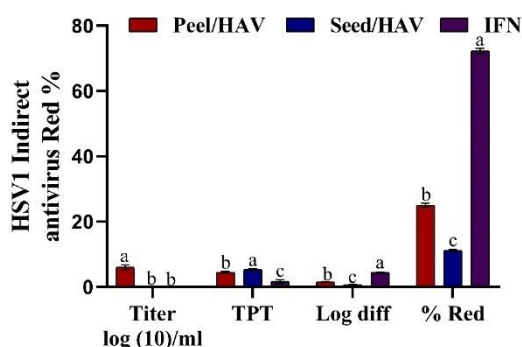


Fig. 2-B. Indirect method antiviral evaluation against herpes simplex virus type-1 (HSV-1). %Red.: Reduction percent, TPT: Titre post-treatment. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

Recorded data revealed that peel extract could induce a 41.17% reduction in HAV titer and a 25 % significant reduction in HSV-1 titer. In comparison, seeds extract significant reduced HAV titer and HSV-1 titer by 29.33% and 11.17%, respectively, when viruses titrated on Vero cell line incubated with extract at 37 °C for 24hr, (Figure 2 A&B).

B-Direct method antiviral evaluation

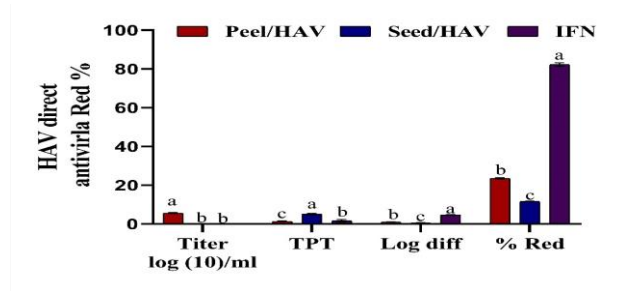


Fig. 3-A. Direct method antiviral evaluation against Hepatitis A virus (HAV). %Red.: Reduction percent, TPT: Titre post-treatment. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

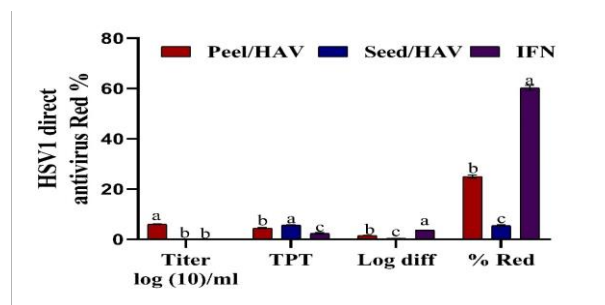


Fig. 3-B. Direct method antiviral evaluation against Herpes Simplex virus (HSV-1). %Red.: Reduction percent, TPT: Titre post-treatment. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

Figure (3A&B) represents the effect of peel extract on HAV and HSV-1 virus titer, which is reduced by 23.50 % and 25.00 %, respectively. In comparison, seed extract reduced HAV titer by 11.66% and reduced HSV-1 titer by 5.50 % when the cell was infected first by viruses and then treated by extracts.

TPT: titer post-treatment

Red.:reductionpercent

c-virucidal activity

Each extract was effective against both viruses as the inactivating agent. The seed extract was highly significant effective within 1 hr against HAV, while both extracts had the same effect within 24 hr, inducing a reduction of virus titer. In the case of HSV-1, the peel extract was highly effective by the time within 18 hr Figures 4(A-B).

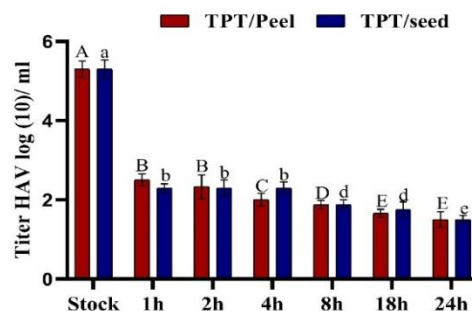


Fig. 4-A. Virucidal evaluation during extracts were incubated with Hepatitis A virus (HAV) virus for a time ranging from 1hr to 24hr histogram. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

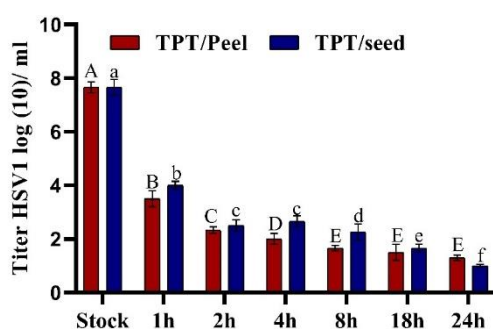


Fig.4-B.Virucidal evaluation during extracts were incubated with Herpes Simplex virus type-1 (HSV-1) virus for a time ranging from 1hr to 24hr histogram. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

d-Plaque reduction assay

Table 3. Indirect method plaque assay

Item	HAV Seed Titer PFU log ₁₀ /ml	HAV titer PFU/ml	HSV-1seed Titer PFU log ₁₀ /ml	HSV-1titerPFU/ml
Peel	3.73*10 ⁵	2.3*10 ²	5.36*10 ⁷	3.15*10 ⁴
Seed		4*10 ⁴		3.73*10 ⁵
IFN		1.05*10 ¹		1.16*10 ¹

Peel extract showed significantly reduced plaques No. Then, the seed was compared with a seed stock of HAV, where there was an insignificantly reduced plaque No. When HSV-1 was treated with both seed and peel, the peel was significantly reduced compared to seed stock. Finally, the IFN used showed significantly reduced plaques. There is no more than that when using peel and seed extracts. Concerning the indirect application of peel and seed extract, Table (3).

Table4. Direct method plaque assay.

Item	HAVSeedTiterPFUlog ₁₀ /ml	HAVtiter PFU/ml	HSV-1 seed Titer PFU log ₁₀ /ml	HSV-1titer PFU/ml
Peel	3.73*10 ⁵	0.93*10 ¹	5.36*10 ⁷	3.15*10 ⁴
Seed		3.5*10 ⁵		3.96*10 ⁵
IFN		1.67*10 ¹		1.67*10 ²

Through the direct method, HAV was significantly reduced ($P < 0.05$) than seed stock and under the effect peel than in the case of using seed-inducing extract. At the same time, HSV was significantly reduced under the effect of both peel and seed extract and insignificantly reduced when treated using both seed and peel extracts ($P < 0.05$). Similarly, IFN-treated viruses showed significantly decreased plaques No. Post application of IFN than that induced using peel and seed extract (Table 4).

3.4. MX-gene expression

Mx gene expression showed a higher highly significant expression rate in the case of PSE-treated cells than in the case of PPE-treated One. In terms of antiviral activity, MX-A gene expression ensured the PPE's antiviral potential in the current investigation. Meanwhile, the MX-A gene is more highly expressed in the case of PSE than in the case of PEE.

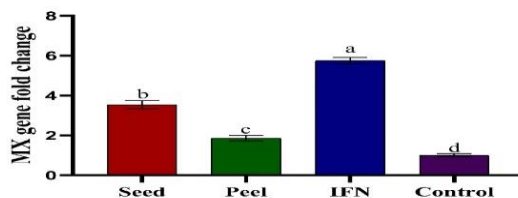


Fig.5.. Assessment of Mx gene expression in Vero cells post-treatment with PPE & PSE and IFN compared to untreated negative control cells. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

3.5. Antioxidant profile

GSH was significantly reduced ($P<0.05$) in the case of both seeds and Peel-treated cells compared with that detected in untreated cell control. Also, the GSH level in seed-treated cells was highly significantly ($P<0.05$) decreased than peel-treated cells (Fig 4). Similarly, there was a highly significant increase ($P<0.05$) in ROS concentration in both seed and peel-treated cells compared with that in untreated cell control. In the meantime, seed-treated cells showed a highly significantly ($P<0.05$) elevated ROS than Peel-treated ones. In the meantime, Lipid peroxidation (MDA) showed the same profile of ROS and NO levels in treated cells (Figures 5, 6 and 7).

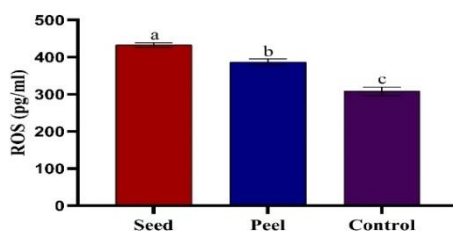


Fig.7.Determination of ROS concentration in Vero cells post-treatment with PPE & PSE compared to the non-treated cell control as negative control. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

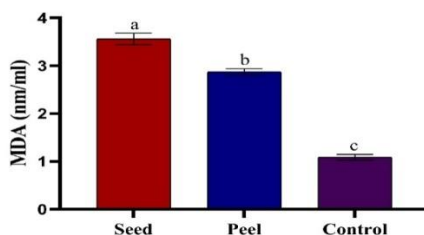


Fig. 8. Determination of MDA concentration in Vero cells post-treatment with PPE & PSE compared to the non-treated cell control as negative control. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

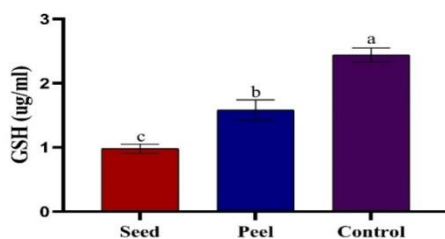


Fig. 6. Determination of GSH concentration in Vero cells post-treatment with pomegranate peel extract (PPE) & pomegranate seed extract (PSE) compared to the non-treated cell control as a negative control. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

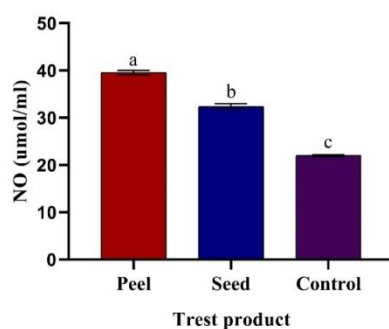


Fig.9. Determination of (NO) concentration in Vero cells post-treatment with PPE & PSE compared to the non-treated cell control as negative control. Statistically, different letters indicate significant differences according to the Fisher test at p-value <0.05.

3.6. docking studies

Viralenzymes

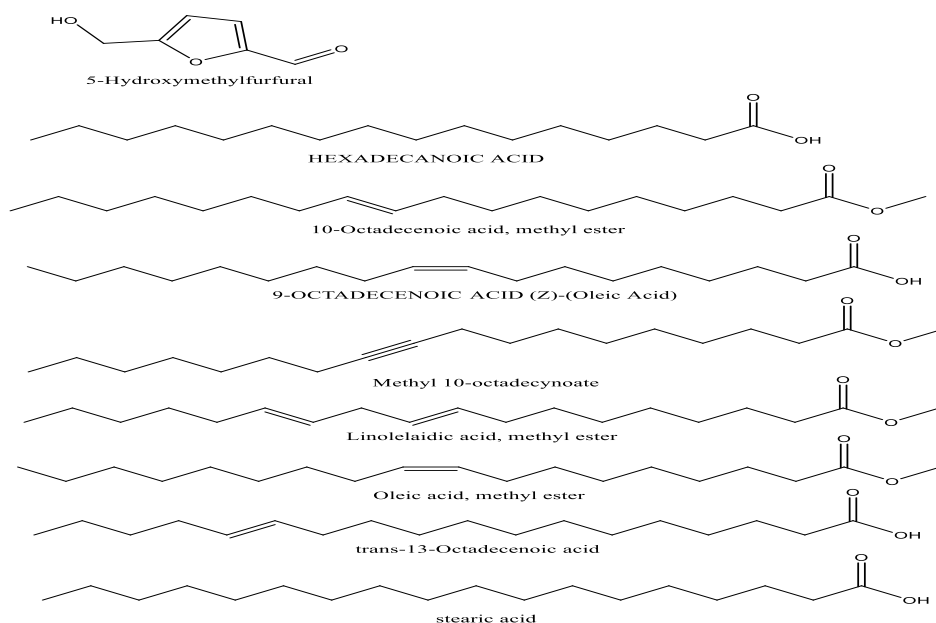
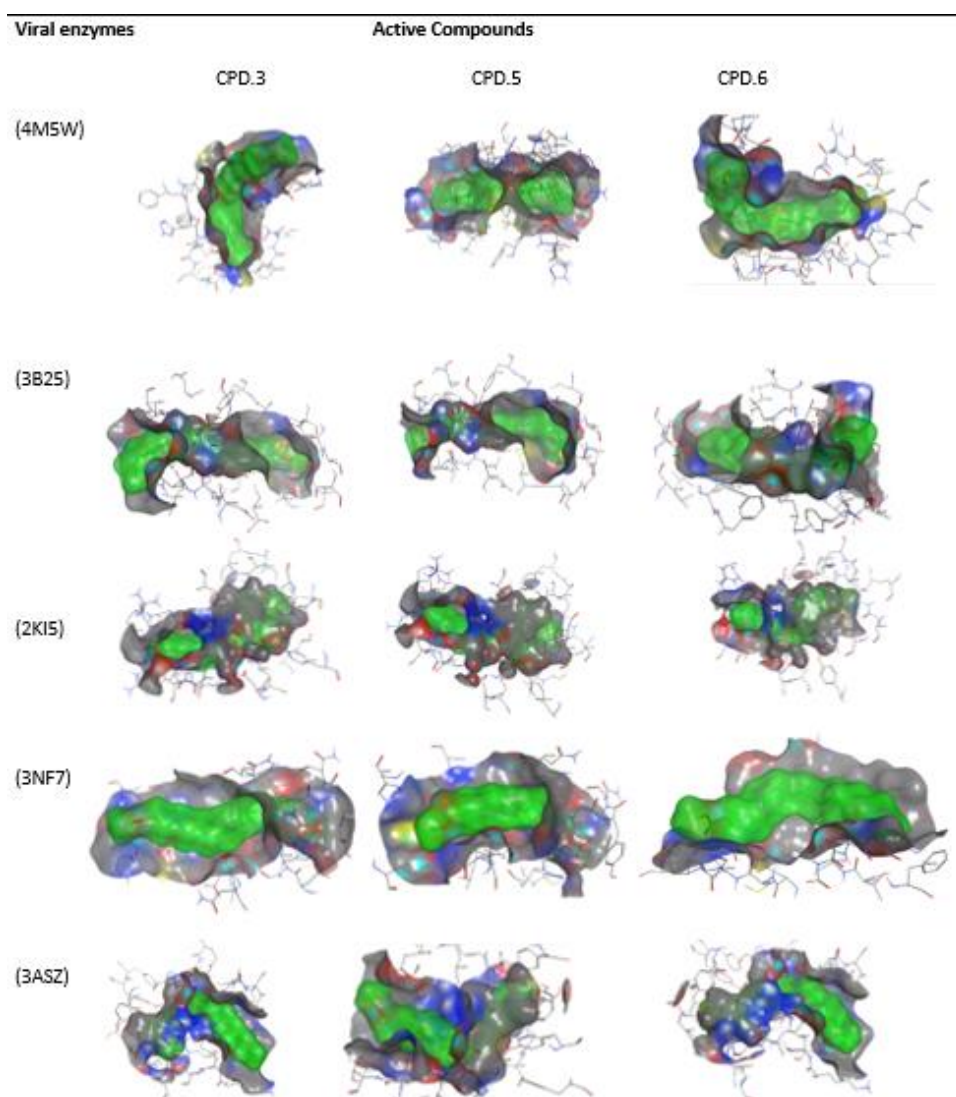


Fig. 10. Selected compounds for docking enzymes

Docking study investigates the Binding mode and ligand interaction of tested compounds inside the active site (PDB ID) of followed viral enzymes:

Table 5. 3D pictures representing the interaction of active compounds (3, 5,6) with the active site of viral enzymes.**Energy table (Estimated free energy) (Kcal/mol) related to binding of ligand and the targeted macromolecules:**

Compound	Energy (4M5W)	Energy (3B25)	Energy (2KI5)	Energy (3NF7)	Energy (3ASZ)
1	-4.6071	-4.4464	-4.7886	-3.8485	-4.9653
2	-6.9067	-7.5000	-6.2054	-5.8039	-5.9547
3	-7.1977	-8.1870	-7.5943	-6.2880	-6.4647
4	-7.2426	-7.1786	-6.9344	-6.2693	-5.6704
5	-7.6921	-8.2832	-8.3212	-6.5157	-6.2810
6	-7.5084	-7.5032	-7.7203	-6.3552	-6.9596
7	-7.3265	-7.6171	-7.4479	-6.1639	-6.8900
8	-7.5767	-7.4605	-6.8373	-6.0728	-6.4245
9	-7.0507	-7.6273	-6.9558	-6.3373	-5.5296

Molecular docking of all the tested compounds were docked in the active site of the enzymes. The results in Table 9 revealed that compound 5 has the highest binding energy to the active site (2KI5) & (3B25), followed by compound 3 to the active site (3B25). The highest binding energy to active sites (4M5W) & (3NF7) was also compound 5, while the highest binding energy to the active site (3ASZ) was compound 6.

4. Discussions

This study aimed to investigate the antiviral potentials of pomegranate peel and seed extracts against elected HSV type 1 and HAV. Plant-based treatments have a long history of being used to cure a variety of ailments, including viral infections and liver diseases. Plant-derived compounds are distinguished from synthetic compounds by their significant chemical variety, drug-like capabilities, biochemical specificity to various viral enzymatic tests, and ability to be absorbed and metabolized by the body with little or no toxicity [26,27].

Furthermore, advances in active compound isolation and characterization methodologies are becoming more beneficial in broad-spectrum screening of a library of plant-derived compounds for antiviral therapy. Chemically identifying compounds in purified pomegranate seed and peel extracts demonstrates the results showing 32 and 39 compounds in seeds and peel extracts, respectively. The main compound among them was 5-Hydroxymethylfurfural, which exhibited antioxidant activity [28,29]. The IC₅₀ records refer to the amount of the extract needed to inhibit the biological Processes of the cell by 50% and also measure the potency of the extract [30]. The results for the cytotoxic activity of peel matched the findings of the previous investigation [31]. Our result showed a noticeable difference in the viability% between peel and seed extracts, which ensures the safety of peel extract over seed extract at high concentrations. This indicates that pomegranate peel extract is safer for Vero cells than seed extract. This was following the report of Salles, et al. [1]. Safety measure of pomegranate peel means the excellent or potent activity of peel extract according to the classification of samples based on IC₅₀ in the previous study [32,33], which agrees that Pomegranate peel extract not only has excellent potency but also improves cell growth.

The microscopic examination indicated the cytotoxic effect of both tested peel and seed extracts on Vero mammalian cells incubated for 24 hr at 37°C. Concerning the antiviral potential of both peel and seed extracts our result was in agreement with Cerdá, et al. [12] despite their *In Vivo* application of extracts recording that they were safe on body organs including the heart, liver, and kidney. Fifty percent cell culture infectious dose (TCID₅₀) endpoint dilution assay and plaque assay are two popular techniques for measuring anti-virus activity [34]. Both assays depend on the ability to measure the observable cytopathic effect (CPE) detected in infected cells. Cytopathic effect means structural modification brought by viral infection of the infected Vero cells, which do not resemble the normal host cell.

CPE appears when the infecting virus leads to host cell lysis or cell death due to its inability to regenerate. The recorded result was in agreement with Su, et al. [35]. Despite the difference in the virus model, they reported that pomegranate extracts exhibited antiviral activity against human norovirus surrogates, HIV-1, influenza, herpes, food-borne viruses, and pox viruses. The current study's results indicated that peel extract's antiviral potential was more effective than seed extract, which was in accordance with Moradi, et al. [36], demonstrated that PPE has strong potential antiviral effects against viruses.

Also, Zhang, et al. [37] reported that pomegranate pericarp tannin has antiviral activity against herpes virus *in vitro* and also can block its adsorption to cells, causing virus death. Also, Rowe and Bolger [38], Moradi, et al. [10] reported that the antiviral potential of pomegranate was extract mode of action dependent, which was in agreement with the records of the present study, where HAV was more sensitive to peel. Seeds extracted from HSV-1 using indirect method antiviral evaluation, while HSV-1 was more sensitive to peel extract than HAV, HAV was more sensitive to seeds extracted than HSV-1 using indirect method. Similarly Su, et al. [35] assured the antiviral potential of pomegranate extracts against hepatitis viruses. In the present study, the virucidal efficacy of both peel and seed extracts was considered, and data recorded revealed that they were virus- and extract-dependent.

It was noted that the virus model titer was significantly reduced within 1 hr which means that viral replication initial stages for both two extracts were inhibited agreeing with virucidal properties by viral replication suppression reported by Haidari, et al. [39] and Moradi, et al. [10] reporting that impeded virus adsorption and the initial stages of viral reproduction were prevented ceasing the synthesis of viral proteins. This supported our reported records that peel extract has a beneficial effect on the cell receptor and virus epitopes interaction while facing the viruses and acts as an excellent anti-virus agent.

The reported previous results were in agreement with our experiments, which indicated that the HAV is more sensitive to the pomegranate peel and seed extracts than the HSV-1. Also, peel extract was highly effective as an antiviral than seed extract against both HAV and HSV-1 due to its rich in bio-active components such as polyphenols, sesquiterpenes, and triterpenes, all of which have antiviral properties as reported by Tito, et al. [40] and Xiang, et al. [41]. The expression of the antiviral activity marker gene (Mx) was evaluated using a safe concentration of both PPE &PSE extracts treated Vero cells. Mx expression became generally acknowledged as a reliable biomarker for type I interferon action [42,43]. The imbalance between reactive oxygen species (ROS) production and enzymatic and nonenzymatic antioxidants causes oxidative stress. Biomarkers of oxidative stress are important in determining disease status and the health-promoting effects of antioxidants [44]. In the current study oxidative stress was evaluated in both PEE and PSE treated and untreated cells, both MDA and ROS were elevated in a test extract-dependent pattern, while GSH was treated cell extract-dependent. GSH acts as a defense system within the cells to prevent uncontrolled ROS production [45], also has an antiviral effect [46,47], and decreases HSV-1 replication.

Recorded data were in agreement with many reports that peel extract contains more antioxidant agents than seeds and pulp [48]. The antioxidant profile showed that pomegranate peel extract has antioxidant properties on HAV & HSV-1, which the defense was in agreement with Arun and Singh [49] and [50]. Pomegranate contains a significant amount of specialized flavonoids, which are powerful antioxidants made up of unusual tannins including punicalagin and other anthocyanins [10]. Nitric oxide (NO) is involved in many physiological functions. Numerous elements of inflammatory responses are mediated by nitric oxide (NO), which is significant in this process. NO is a chemical that has antioxidant properties. It can control the release of different inflammatory mediators from a variety of cells involved in inflammatory reactions [51].

Molecular docking investigated the interactions between ligands and receptors of compounds (1-9) derived from extracts of pomegranate seeds and peel, focusing on their binding affinity with the active sites of viral enzymes. The findings revealed that all compounds examined exhibited a notable affinity for the active site, with binding energies ranging from (-3.8485 to -8.3212) kcal/mol. The calculated standard binding free energies were found to be consistent with previously reported experimental data [16]. The binding of a compound to the active site of an enzyme leads to a competitive interaction with the substrate, resulting in the formation of an enzyme-inhibitor complex that hinders the enzyme's catalytic activity until the inhibitor dissociates, a phenomenon commonly referred to as competitive inhibition.

5. Conclusions

Both the peel and seed extracts exhibit potential antiviral properties against hepatitis A virus (HAV) and herpes simplex virus type 1 (HSV-1). The peel extract not only surpasses the seed extract in terms of safety but also demonstrates significant antiviral activity within cells. The superior antiviral efficacy of the peel extract against the test viruses was found to be independent of the MX-A gene, unlike the seed extract. This disparity can be attributed to the distinct composition of the peel extract, as revealed by GC-MASS analysis. Moreover, the peel extract is richer in antioxidant compounds than the seed extract. A docking study identified compounds such as 10-octadecanoic acid methyl ester, methyl-10-octadecynoate, and linolelaidic acid methyl ester as active agents against both HAV and HSV-1. Overall, pomegranate peel shows great promise in terms of therapeutic potential and virucidal effects against HAV and HSV-1.

6. Recommendations

Future research is suggested to explore the potential utilization of pomegranate peel extract in various herbal remedies instead of the seeds or the whole pomegranate extract due to its heightened efficacy. This enhanced effectiveness might be associated with different genes apart from the MX-A gene. The process of docking studies involves the identification of the top three scoring compounds (compound 3 found in seeds and compounds 5 and 6 found in the peel) based on their calculated free energy of binding to the active sites of viral enzymes, which are then selected for further detailed examinations.

7. Conflicts of interest

“There are no conflicts to declare”.

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