Arab Universities Journal of Agricultural Sciences
(2024) 32 (1) 53-63
Website: http://ajs.journals.ekb.eg

In Silico and *In Vitro* Assessment of Antiviral Activities of Licorice and Green Tea Extracts Against Tobacco Mosaic Virus

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https://doi.org/ 10.21608/AJS.2024.255283.1543

Received 21 December 2023 ; Accepted 20 March 2024

Keywords:

Licorice,
Green tea,
Antiviral activities,
TMV,
Molecular docking

Abstract: Using integrated *in silico* and *in vitro* experiments, the antiviral capabilities of green tea and licorice extracts were evaluated against the tobacco mosaic virus (TMV). It was noted that the number of induced necrotic local lesions of TMV decreased by increasing extract concentrations and thus it is considered concentration dependent on the extracts' active compounds. Properties of eleven compounds belonging to licorice and green tea were obtained from databases to examine their activities *in silico*. A molecular docking simulation was performed between these agents and the protein coat of the tobacco mosaic virus (TMV). The highest effective compounds were glycyrrhetic acid, liquiritin and EGCG. ADMET studies revealed good overall properties of nine non-toxic compounds based on their predicted pharmacokinetic, physicochemical, drug-likeness and toxic properties. Based on docking energy and mode of interaction, these compounds showed strong binding with the protein coat of TMV. The antiviral effect may be due to the most effective compounds with the highest affinity namely glycyrrhetic acid, liquiritin and isoliquiritin. As a conclusion, the promising value of our titled extracts is the anti-TMV activity and pharmacokinetic and physicochemical properties of their active constituents.

1 Introduction

Since the 1950s, attention has been paid to the importance of natural antiviral agents. Some studies were conducted on the use of biological antiviral agents for virus control. Recently, the use of biological antiviral agents has significantly increased (Abonyi et al 2009). Medicinal plants are frequently used as raw materials for the extraction of active ingredients which are, in turn, used in the synthesis of different drugs (Hassan 2012). They are found to be important sources of natural compounds as antiviral agents (Jansi et al 2021).

Several investigations reported that no effective commercial antiviral pesticides were available to completely control viral diseases (Li et al 2017, Wang et al 2019 a and b). Therefore, the use of medicinal plant extracts as antiviral agents that enhance defense-related gene expression could be a suitable alternative to control some plant viruses (Zhao et al 2017). As a result of

extensive studies, some progress has been made and effective antiviral agents extracted from some medicinal plants have been designed and used to protect plants from viral diseases (Ai et al 2010, Wang et al 2012, Wang et al 2014, Sofy et al 2021).

Plant viruses cause severe economic damage to crops, which greatly threatens the agricultural industry and its development (Rubio et al 2020, Jones 2021, Mehetre et al 2021).

The tobacco mosaic virus (TMV) was known as the most suitable model for studying the characterization and identification of plant viruses. The virus was classified as a member of the genus *Tobamovirus*, Family *Virgaviridae* (Knapp and Lewandowski 2001, Adams et al 2017, Lomonossoff and Wege 2018). The virus was considered one of the most serious phytopathogens infecting several crops of economic importance such as tomato (Hussain et al 2018) as well as a wide range of about 400 economically important plants, including *Solanaceae* plants (tobacco, potato and pepper), vegetables and ornamental flowers species (Scholthof 2004, Roossinck and García-Arenal 2015, Chen et al 2016, Adams et al 2017, Gan et al 2017a and b, Guo et al 2019).

TMV is known as plant cancer due to the deformation of fruits and leaves and dwarfing of plants, which reduces seed germination rates (Su et al 2014, Lv et al 2020). The importance of TMV-protein (RdRp) was reported as an important protein for controlling TMV infection since the TMV-RdRp domain is necessary for the inhibition of TMV complex by providing the catalytic activity in charge of synthesizing TMV RNA plus mediating TMV replication (Xu and Zhang 2011, Hussain et al 2018).

A number of agrochemicals obtained as natural products have been evaluated for use as active agents against TMV, for the sake of finding possible alternative pesticides that do not pose environmental threats (Wang et al 2012, Wu et al 2013, Wang et al 2014). Regarding TMV, a few *in silico* studies have been carried out (Zhu et al 2019, Nagalakshmamma et al 2020, Wang et al 2020). Antiviral activity of the essential oil of *Melaleuca alternifolia* L. (tea tree) against TMV was evaluated *in vitro* on *Nicotiana glutinosa* L. plants as a pre-inoculation spray at different concentrations (100, 250 and 500 ppm). The number of necrotic local lesions (NLL) produced by TMV on *N. glutinosa* leaves pre-treated with the essential oil of *M. alternifolia* before virus inoculation decreased ten days post-inoculation (Bishop 1995).

Both molecular docking and molecular dynamics together as important therapeutic methods, in drug design and discovery, were successfully used to evaluate the type of binding between the ligand and its enzyme receptor (Dunigan and Zaitlin 1990). Abdulhassan et al (2022) evaluated the activity of a selection of 41 novel and 2 reference standardized compounds against anti-TMV and examined the interaction and efficiency of these compounds in binding to the TMV envelope protein. There was an urgent need for a new anti-TMV agent that could prevent such a disease; that is why several recent studies aimed to evaluate the anti-TMV anti-*silico* activities of 43 anti-vin analog compounds, including ningnanmycin 1 and ribavirin 2. Selected compounds with diverse activity against TMV were separated (Wang et al 2012). To achieve this goal, the interaction of these compounds and their binding mode to the TMV-CP were simulated using Gaussian 09 software with B3LYP/6-31G base group and MOE software and then compared with standard anti-TMV agents such as ningnanmycin 1 and ribavirin 2 (Vilar et al 2008). Wang et al (2020) reported bioassay assessment of several compounds showed good antiviral activities against TMV. They also conducted docking analysis against TMV in a trial to identify the potential ligand sites responsible for the inhibition of TMV replicase.

Opo et al (2021) identified the possible natural antagonist against the X-linked inhibitor of apoptosis protein (XIAP), a protein for cancer treatment, based on the computer-aided drug design process, *i.e.*, virtual screening, dynamic simulation, molecular docking, pharmacophore modeling and ADMET. Different types of XIAP antagonists were applied to repair the defective apoptosis process that could eliminate carcinoma from living bodies.

Based on several reported studies on some medicinal plants having antiviral activities, it was found that both licorice (Pastorino et al 2018) and green tea (Reto et al 2007) were active remedies against different human viruses. Therefore, this study aims to conduct *in silico* and *in vitro* assessments of the antiviral activities of licorice and green tea extracts against the plant virus TMV.

2 Materials and Methods

2.1 Source of medicinal plants

Licorice roots and green tea leaves were obtained from the Herbalist shop in Cairo. Licorice roots were well sun-dried before being used.

2.2 Determination of antiviral activities of liquorice and green tea extracts against TMV

A weight of five grams of sun-dried licorice roots and green tea leaves were ground into a powder form. Afterward, Maceration method was used which involved soaking plant powder in a stoppered container with 70% ethanol as well as sterilized distilled water then was allowed to stand at room temperature for one week. The extract was collected and filtered followed by drying using a rotary evaporator. The powder form was re-suspended into 10 mL sterilized distilled water and stored at 4°C for further use. A number of 55 treatments were designed and carried out to assess the antiviral activities of liquorice and green tea extracts against TMV under greenhouse conditions.

2.3 TMV isolate and its confirmation

Young tobacco plants (*Nicotiana tabacum* cv. Turkish) with 4-5 leaves were selected as they give a sufficient amount of juice containing TMV particles. The extract was prepared according to the method described by Kassanis and Milne (1971). TMV was mechanically inoculated on some diagnostic hosts by contact and rubbing of the viral-infectious sap on the surface of the lower or middle leaves in the presence of an abrasive substance such as smeared carborundum an abrasive material according to the procedure of Kassanis and Milne (1971). Infection was performed on plants belong to different plant families, *i.e*., Chenopodioideae (*Chenopodium amaranticolor*), Solanaceae (*Datura metel*, *Datura stramonium*, *Nicotiana glutinosa*, *Nicotiana tabacum* cv. Turkish and *Nicotiana tabacum* cv. Samsun NN). Plants were grown under greenhouse conditions.

2.4 Inclusion bodies of TMV

Light microscopy inspection was carried out on living cells from epidermal strips of the undersides of Turkish tobacco leaves 10 days post TMV inoculation. According to the method of Kassanis and Milne (1971), each of the crystalline and amorphous inclusion bodies induced by TMV was detected by light microscopy of some strips from TMV-infected *Nicotiana tabacum* cv. Turkish exhibited systemic infection 10 days post TMV-mechanical inoculation. Regarding amorphous inclusions, the strips post-treated with 1% triton X-100 were stained with 1% trypan blue stain for 20 minutes before examination.

2.5 *In silico* assessment of antiviral activities of licorice and green tea extracts against TMV

Antiviral activities of licorice and green tea active compounds were examined against TMV by predicting protein-ligand binding affinities. The structure of the protein coat of the TMV was downloaded from the protein data bank (PDB ID: 3KML). Nine non-toxic active compounds, two of licorice (glycyrrhetic acid and liquiritin) and seven of green tea [caffeine, epicatechin (EC), epicatechin gallate (ECG), catechin, gallocatechin, epigallocatechin gallate (EGCG) and epigallocatechin (EGC)] were docked using CB-Dock server (https://cadd.labshare.cn/cb-dock2/).

The toxicities of these compounds were determined based on their pharmacokinetics (GI absorption, BBB permeant, P-gp substrate, CYP1A2 inhibitor, CYP2C19 inhibitor, CYP2C9 inhibitor, CYP2D6 inhibitor, and CYP3A4 inhibitor) and physiochemical (Formula, Molecular weight, (g/mol), No. of heavy atoms, No. of rotatable bonds, No. H-bond acceptors and No. of H-bond donors). Properties were assayed by ADMET prediction using SwissADME server (http://www.swissadme.ch/) (Daina et al 2017).

2.6 Mode of interaction of most active compounds

Binding affinity, docking score, interacting amino acids and type of bonds were determined. 2D images were visualized between ligand and protein using Discovery studio client [https://discover.3ds.com/discovery-studio-visualizer-download].

3 Results and Discussion

3.1 *In vivo* assessment

The TMV isolate was varied in its reaction when inoculated on different diagnostic hosts as reported by several studies (Herold and Munze 1967). It showed systemic infection (mosaic, blisters and deformation) on *Nicotiana tabacum* cv. Turkish while it gave NLL on each of *Chenopodium amaranticolor*, *Datura stramonium*, *Datura metel*, *Nicotiana glutinosa*, and *Nicotiana tabacum* cv. Samsun NN. These results are in harmony with those reported by Setoyama et al (1993), Klarzynski et al (2003) and Ruíz del Pino et al (2003). It is well known that TMV, one of the most economic viruses affecting several plant species, can be characterized by inducing two types of inclusion bodies, *i.e.*, crystalline and amorphous in the cytoplasm of infected plant cells, in particularly tobacco plants that show systemic infection represented by mosaic, vein banding, blisters and deformation (Christie 1967, Knapp and

Lewanadowski 2001). Light microscopy of stripes showed the presence of paracrystalline of various shapes in hairs, epidermal cells and hexagonal crystals**.** Examination of stripes stained with 1% trypan blue showed inducing amorphous granule inclusions stained with light blue compared to the nucleus which appeared with a dark blue color**.** These results agreed with those reported by Chandra and Hildebrandt (1967) who mentioned that TMV-infected cells frequently had hexagonal crystals, gray plates, and paracrystalline needles in different shapes. Their findings supported the experimental results in particularly inducing crystalline and amorphous inclusions by TMV in the cytoplasm of tobacco plant leaves (Epidermal and hair cells).

Antiviral effects of plant extracts against several plant viruses have been reported (Iftikhar et al 2013, Rasoulpour et al 2017). Liquorice was found to contain active compounds (glycyrrhizic acid, glabridin and liquiritin) having antagonistic activities against different plants and human pathogens while liquiritin, the main flavonoid found in liquorice root, has antiviral effects (Kim et al 2006).

The antiviral activity of liquorice and green tea ethanolic extracts against TMV was estimated and presented in **Table 1** and illustrated by **Figs 1 and 2** respectively. Green tea extract was more efficient than liquorice extract since the number of NLL of green tea extract at the highest dilution (1/512) was 70 with an inhibition rate of 49.28%, while it was 78 with an inhibition rate of 43.48% in the case of liquorice extract at the same dilution **Table 1.** The experimental results are in harmony with those reported by Rasoulpour et al (2017), who emphasized the investigation of the antiviral effects of liquorice extract in locally infected hosts based on the number of NLL induced by TMV on *N. glutinosa*.

It is worth mentioning that the number of NLL induced by TMV on datura plants (*D. metel* or *D. stramonium*) used to be more than that induced by the same virus, *i.e*., TMV on tobacco plants, such as tobacco Samsun NN; this is due to that datura plant was noted in several investigations to be more hypersensitive to TMV infection than tobacco plants. Besides, most tobacco plants used to appear systemic infection when inoculated with TMV strains while datura plants used to show local infection with all TMV strains (Kassanis and Milne 1971). Therefore, results in **Table 1** showed a number of NLL induced by TMV on *Datura metel* plant (195) higher than that induced on *Nicotiana tabacum* cv. Samsun NN (138) when inoculated with crude viral sap.

Similar observations were recorded when ethanol extract was used with the same dilutions. It is worth mentioning that green tea ethanol extract showed higher antiviral activities against TMV than those of liquorice ethanol extract at the same levels.

3.2 *In silico* assessment

Searching indicated the presence of eleven compounds belonging to licorice (Pastorino et al 2018) and green tea (Reto et al 2007) proved to have antagonistic activities against different viruses. Out of these compounds, two non-toxic compounds were belonging to licorice (glycyrrhetic acid and liquiritin), and seven were belonging to green tea extract [caffeine, epicatechin (EC), epicatechin gallate (ECG), catechin, gallocatechin, epigallocatechin gallate (EGCG) and epigallocatechin (EGC)].

Reported bioactive agents were docked against the coated protein of TMV along with the reference drug acyclovir; docking results were elucidated based on their interaction energies (**Table 2, Fig 3)**. The reference drug, acyclovir showed a binding affinity of -5.7 kcal/mol while all other 11 compounds had higher binding affinities against TMV where their binding affinity ranged from -6.2 to -8.7 kcal/mol. The highest effective compound was glycyrrhetic acid followed by liquiritin and isoliquiritin with binding energies of -8.7, -8.2, and -7.9 kcal/mol, respectively.

Further analysis for the binding modes of the most active agents glycyrrhetic acid, liquiritin and isoliquiritin as well as acyclovir are summarized in **Table 3.** The Acyclovir bonded through H-bonds to three essential amino acids namely, ARG15, ALA144 and THR151, it also revealed one alkyl interaction with ALA19. Glycyrrhetic acid showed two types of bonds with four amino acids of TMV protein namely alkyl interaction with the steroid moiety to VAL21, ALA144 and ALA26, the fourth bond was due to conventional H-bond between ASN29 and the carboxylic group of the compound.

Liquiritin interacted with nine amino acids, the sugar moiety of the compound bonded to PRO140, ASN28, CYS25 and ALA144 through five H-Bonds while the chromone ring bonded to THR151 through H-Bond and to ALA22 and ALA19 through hydrophobic interaction. For Epigallocatechin gallate (EGCG), four types of bonds were found with many amino acids namely ARG15, ASP17, ASN28, GLN98, and THR151; Alkyl and Pi-Alkyl interactions with amino acid ALA148 were revealed. Alkyl interaction with ALA22 and finally carbon-hydrogen interaction with

Table 1. Antiviral activities of licorice and green tea extracts against TMV.

Water extract				Ethanol extract			
Treatments (ppm)		Average No. of NLL	% Ant-virus activity	Treatments (ppm)		Average No. of NLL	% Ant-virus activity
Licorice				**Licorice**			
T01	LWE (25000)+TMV infectious sap	06	95.65	T19	LEE (25000)+TMV infectious sap	25	87.18
T02	LWE (12500)+TMV infectious sap	11	92.03	T20	LEE (12500)+TMV infectious sap	35	82.05
T03	LWE (6250)+TMV infectious sap	17	87.68	T21	LEE (6250)+TMV infectious sap	70	64.10
T04	LWE (3125)+TMV infectious sap	21	84.78	T22	LEE (3125)+TMV infectious sap	75	61.54
T05	LWE (1563)+TMV infectious sap	39	71.74	T23	LEE (1563)+TMV infectious sap	85	56.41
T06	LWE (781)+TMV infectious sap	45	67.39	T24	LEE (781)+TMV infectious sap	105	46.15
T07	LWE (391)+TMV infectious sap	54	60.87	T25	LEE 391)+TMV infectious sap	118	39.48
T08	LWE (195)+TMV infectious sap	67	51.45	T26	LEE (195)+TMV infectious sap	128	34.36
T09	LWE (98)+TMV infectious sap	78	43.48	T27	LEE (98)+TMV infectious sap	132	32.31
T10	GTWE (25000)+TMV infectious sap	04	97.10	T28	GTEE (25000)+TMV infectious sap	15	87.69
T11	GTWE (12500)+TMV infectious sap	12	91.30	T29	GTEE (12500)+TMV infectious sap	30	84.61
T12	GTWE (6250)+TMV infectious sap	20	85.50	T30	GTEE (6250)+TMV infectious sap	58	70.25
T13	GTWE (3125)+TMV infectious sap	25	81.88	T31	GTEE (3125)+TMV infectious sap	63	67.92
T14	GTWE (1563)+TMV infectious sap	32	76.81	T32	GTEE (1563)+TMV infectious sap	73	62.56
T15	GTWE (781)+TMV infectious sap	34	75.36	T33	GTEE (781)+TMV infectious sap	81	58.46
T16	GTWE (391)+TMV infectious sap	45	67.39	T34	GTEE (391)+TMV infectious sap	89	54.36
T17	GTWE (195)+TMV infectious sap	56	59.42	T35	GTEE (195)+TMV infectious sap	105	46.15
T18	GTWE (98)+TMV infectious sap	70	49.28	T36	GTEE ((98)+TMV infectious sap	125	35.90
Control treatments							
T37	CLWE+TMV infectious sap	42	78.46	T41	CGTWE+TMV infectious sap	06	96.92
T38	CLEE+TMV infectious sap	30	84.62	T42	CGTEE+TMV infectious sap	02	98.97
T39	CLWE+CLEE+TMV infectious sap	15	92.31	T43	CGTWE+CLEE+TMV infectious sap	05	97.44
T40	CLWE+CGTWE+TMV infectious sap	13	93.33	T44	CLEE+CGTEE+TMV infectious sap	01	99.49

Control treatments	No. of treated leaves	No. of infected leaves	Average No. of NLL	% Ant-virus activity
T45: LEE+TMV infectious sap 9 days post extract inoculation on *D. metel*	21	20	2.52	98.70
T46: GTEE+TMV infectious sap 9 days post extract inoculation on *D. metel*	18	17	3.88	98.01
T47: LEE&GTEE+ TMV infectious sap 9 days post extract inoculation on *D. metel*	15	14	2.80	98.56
T48: TMV infectious sap+LEE two hours post virus inoculation on *D. metel*	10	07	2.10	98.92
T49: TMV infectious sap+GTEE two hours post virus inoculation on *D. metel*	10	08	2.50	98.72
T50: TMV infectious sap+LEE&GTEE two hours post virus inoculation on *D. metel*	10	09	2.30	98.82

General control treatments	Average No. of NLL	General control treatments	Average No. of NLL	% Ant-virus activity
T51: Crude TMV infectious sap on *D. metel* diagnostic host	195	T54: Crude GTEE on D. metel diagnostic hostT	000	000
T52: Crude TMV infectious sap on *N. tabaccum* cv. Samsun NN diagnostic host	138	T55: Crude LEE+GTEE on D. metel diagnostic host	000	000
T53: Crude LEE on *D. metel* diagnostic host	000			

NLL: Necrotic local lesions. LWE: Licorice water extract. LEE: Licorice ethanol extract. GTWE: Green tea water extract. GTEE: Green tea ethanol extract. CLWE : Crude licorice water extract. CLEE : Crude licorice ethanol extract. CGTWE : Crude green tea water extract. CGTWE: Crude green tea ethanol extract.

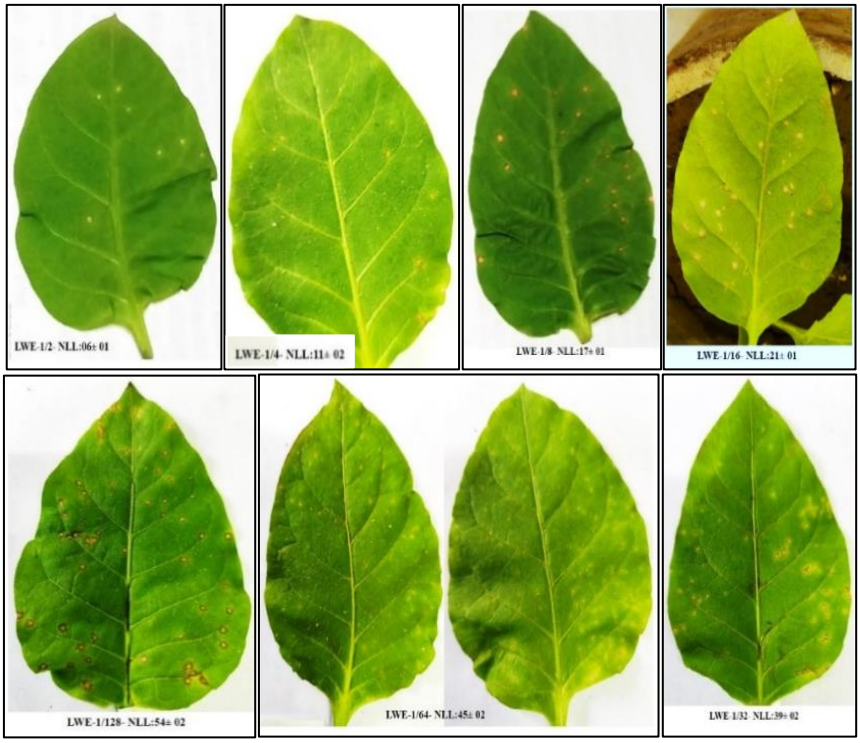

Fig 1. Number of NLL on *Nicotiana tabacum* cv. Samsun NN plant leaves treated with different dilutions of licorice water extract against TMV

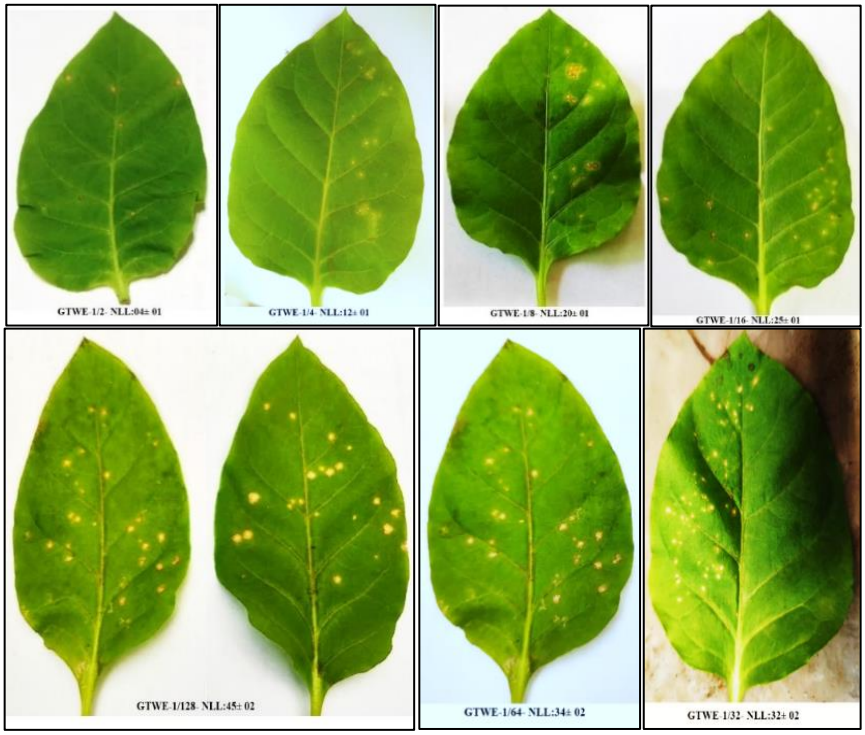

Fig 2. Number of NLL on *Nicotiana tabacum* cv. Samsun NN plant leaves treated with different dilutions of green tea water extract against TMV

Table 2. Antiviral activities of licorice and green tea compounds against TMV based on virtual screening

Source of plants	Effective compounds	Activities	Binding affinities
Liquorice (4 compounds)	Glycyrrhetic acid	✓	-8.7
	Liquiritin	✓	-8.2
	Isoliquiritin	✓	-7.9
	Glabridin	✓	-7.6
Green tea (7 compounds)	Caffeine	✓	-6.2
	Epicatechin (EC)	✓	-6.8
	Epicatechin gallate (ECG)	✓	-7.7
	Catechin	✓	-6.7
	Gallocatechin	✓	-6.9
	Epigallocatechin gallate (EGCG)	✓	-7.9
	Epigallocatechin (EGC)	✓	-6.8
✓= Active.	TMV-control (Acyclovir)		-6.0

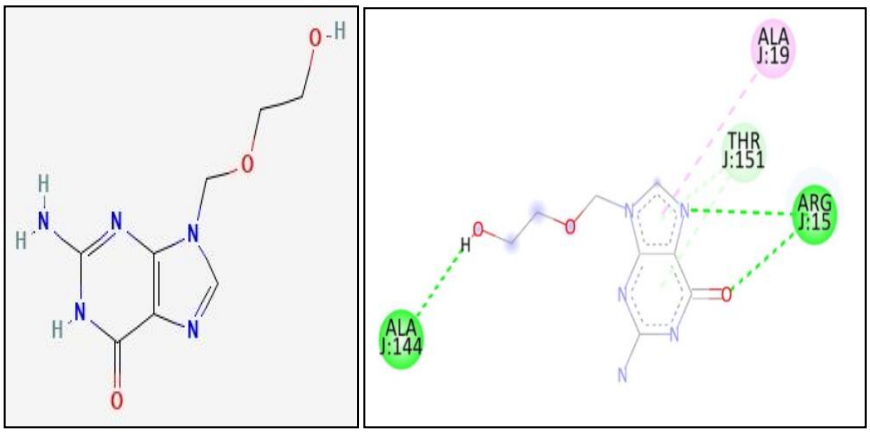

Fig 3. 2D structures of acyclovir and its mode of binding with the coated protein of TMV.

ARG 42 was shown. Most interactions happened between the previously mentioned amino acids and both trimethoxy phenyl rings.

It was noted that the three active derivatives shared similar binding to the reference drug where all three derivatives bonded to ALA144, while liquiritin and epigallocatechin gallate (EGCG) shared a similar interaction to acyclovir through THR151. All the previous results would shed some light on the mechanism of action of the three agents against TMV.

3.3 ADMET Studies

ADMET properties (Absorption, distribution, metabolism, extraction and toxicity) of the active agents were explored to assess the drug-likeness of the compounds including pharmacokinetics and physico-chemical properties, as predicted by the SWISS ADME server. Glycyrrhetic acid, isoplumbagin, glabridin, caffeine, gallocatechin and epigallocatechin (EGC) were predicted as orally available compounds. In addition, all agents were found to be in context with "Lipinski's rule of five" active compounds except for Epicatechin (EC).

It was observed that all seven compounds of green tea were found to be non-toxic compounds. On the other hand, two out of the four licorice compounds (isoliquiritin and glabridin) were found to be toxic.

Table 3. Binding affinities and mode of interaction of most active compounds of licorice and green tea based on docking scores

Plants	Selected non-toxic compounds	Binding energy	Amino acids	Type of bonds
Liquorice	Glycyrrhetic acid	-8.7	VAL21	Alkyl
			ALA144	Alkyl
			ALA26	Alkyl
			ASN29	Conventional H-Bond
	Liquiritin	-8.2	PRO140	Conventional H-Bond
			ALA144	Carbon
			ALA148	Van der Waals
			THR151	Conventional H-Bond
			ALA22	Pi-Alkyl
			ALA19	Pi-Alkyl
			GLY147	Amide-Pi-Stacked
			CYS25	Conventional H-Bond
			ASN28	Conventional H-Bond
Green tea	Epigallocatechin gallate (EGCG)	-7.9	GLN98	Conventional H-Bond
			ASP17	Conventional H-Bond
			ARG15	Conventional H-Bond
			THR151	Conventional H-Bond
			ASN28	Conventional H-Bond
			ALA144	Alkyl & Pi-Alkyl
			ALA22	Alkyl
			ALA148	Alkyl & Pi-Alkyl
			ARG42	Carbon H-Bond
Control	Acyclovir	-5.7	ALA144	Conventional H-Bond
			ARG15	Conventional H-Bond
			THR151	Pi-donor H-Bond
			ALA19	Alkyl

4 Conclusion

Licorice and green tea proved to have antagonistic activities against TMV. The antiviral activities of prepared extracts were evaluated among a number of 55 treatments. Nine compounds, two of licorice and seven of green tea were found to be non-toxic compounds and have antiviral activities against TMV. The binding energy and mode of interaction of these compounds showed good binding with the TMV coat protein. The highest affinity of antiviral effect was found for glycyrrhetic acid and liquiritin compounds.

Acknowledgments

The authors would like to thank the Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt for providing the greenhouse room, and facilities.

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