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IN-VITRO ANTIVIRAL ACTIVITY OF TIRYAQE WABAI AGAINST HERPES SIMPLEX VIRUS-1

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ABSTRACT: Background: Herpes simplex virus (HSV) are one of the most important viruses that causes a variety of life-threatening diseases in humans and the chemotherapeutic agents available for HSV infections are limited in efficiency. The formulation from Unani system of medicine was taken for antiviral assay which has already been established as anti-oxidants, immuno-modulator, anti-inflammatory activity in order to not only to reduce the viral-infection but also to maintain the homeostasis. **Objective:** Tiryaqwabai (TW) is a formulation in Unani system of medicine which was used by traditional healers and scholars like Avicenna and Galen in healthy and in sick people for its prevention and therapeutic effects during epidemics like cholera, plague and other diseases. Antiviral activity of the three components have been established in-vitro against HSV-1. The study was done to evaluate the synergistic effect of the formulation for HSV-1. **Methods:** TW was screened for its cytotoxicity against Vero cell line by microculture tetrazolium (MTT) assay. Antiviral properties of Tiryaqwabai (TW) were determined by cytopathic effect inhibition (CPE) assay and Virucidal assay (virus yield reduction assay.) some more detail is needed here. **Data Analysis:** In the *in-vitro* assays, the number of plaques formed in infected cells was used to calculate the percentage inhibition of plaques and the graphical determination of EC_{50}. In the cytotoxicity experiments, the number of viable cells was used to calculate the percentage toxicity at each tested concentration, which was then used for the graphical determination of the CC50. **Results:** TW powder was found to be non-toxic below 2000mcg/ml and the CPE inhibition assay at 1000mcg/ml and 500mcg/ml have shown 75 +4.45 % and 30.73+2.72% protection against HSV-1 virus challenge dose 10TCID50. In the virucidal assay TW has shown antiviral activity on HSV-1 titration as 99%. **Conclusion:** Tiryaqwabai includes all three components that provide a suitable basis for the preventive application of various pharmacological measures, such as immunomodulatory, atithusic, sputum and antivirals, in infectious conditions. Further investigations utilising more sophisticated techniques are necessary to get potential anti-HSV medicines from the bioactive components extracted from the plants.

INTRODUCTION: With the increase in viral-infections and drug resistance around the globe, there is a need to search for new and more effective antiviral agents. Herpes simplex virus is an enveloped virus that causes gingivostomatitis, herpes labialis, encephalitis, ocular and genital infection, meningitis and pneumonitis [1].

This virus can produce latent infection in the host for life and is reactivated by stimulus to cause recurrent infections and lesions [2]. Acyclovir is the standard treatment used for the treatment however the severe side effects and the development of

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some resistant mutants of this virus, especially during long-term medication with antiviral drugs were reported [3]. Henceforth, traditional medicines ought to be investigated as novel antiviral agents, as many of these ancient medicaments, containing different plant metabolites, have potent antiviral activities [4]. Unani system of medicine is one among the oldest system of medicine derived from animal, plant and mineral sources [5].

Tiryaq-e-wabai and its Importance: The history of tiryaq is around 2000 years old and has been regarded as a universal antidote [6]. As the name suggests, tiryaq means antidote, which is a famous unani complex poly herbal compound preparation used to treat poisons and diseases during epidemics and various disorders. Wabayi means epidemics and pandemics which spreads in a large geographical area [7]. Tiryaqwabai is a Unani polyherbal preparations made up of three ingredients, which was widely used by famous scholars Avicenna and Galen as prophylaxis and treatment in patients during epidemics. It consists of three ingredients Zafran (*Crocus sativus* L.), Mur (*Commiphora myrrha* (Nees) Engl.) and Sibr (*Aloe barbadensis* Mill), in the ratio of 1:1:2 [8].

Pharmacological studies done on Aloe have shown antiviral, anti-inflammatory, antioxidant, anti-asthma, Analgesic and immunomodulatory activity. Antiviral activity against HSV-2 replication in vero-cell line was done using crude hot glycerine extract of *Aloe vera* gel. And has shown to be effective before attachment and various stages of post attachment of virus replication [9]. *Aloe barbadensis* Mill. increases the proliferation of T lymphocytes in involuted thymus. *Aloe barbadensis* Mill. found to have Antiflu, Antibacterial, wound healing-promoting and immunity-enhancing functions, Anti-inflammatory and antioxidant [10]. Studies on effect of crocin and safranal showed suppression of inflammatory pain responses and anti-inflammatory effect. Zafran (*Crocus sativus* L.) pharmacological studies presented the effects such ad anti-tussive, anti-microbial, antioxidant, anti-hyperglycaemic, anxiolytic, anti-convulsant, hepato-protective, cytotoxic and anti-inflammatory activity [11]. The active constituent of saffron are Safranlal, Picrocrocin, crocetin and crocin, which are useful in the treating ailments due to neurogenerative disorders. The antitussive effect of stigma and petals of saffron and crocin in guinea pigs have been evaluated in study [12]. Antihyperglycemic activity, anticoagulant activity, cell proliferation inhibition, tranquillizing of constituents of Saffron extracts have been reported in the study conducted [13].

Murmakki (*Commiphora myrrha* (Nees) Engl.) is an effective combination because of its underlying properties antimicrobial, Antiseptic, anti-inflammatory, bacteriostatic, Antiviral and Antiviral and leucocytogenic agent [13]. Myrrh can be used to treat cold and to relieve nasal congestion. The extracts of *Commiphora myrrha* (Nees) Engl. was documented to treat the MCF-7 breast cancer cell line. The Antioxidant effects are a possible mediator in the protection against myocardial necrosis, inhibition of platelet aggregation, and increased fibrinolysis by extract of myrrh resin. It was reported to be effective in fascioliasis and in the treatment of Schistosomiasis haematobium in animal studies [14]. The methanolic extract of Mur Makki (*Commiphora myrrha* (Nees) Engl) has shown antimicrobial activity against *E. coli, S. aureus, B. cereus* and *K. pnuemoniae*. The petroleum ether extract showed effective results against *S. aureus, E. coli, Pseudomonas aeruginosa* and anti-fungal activity against Aspergillus species and *Candida albicans* [15].

The Unani polyherbal formulation (Tiryaqewabai) has shown to be immune stimulating among immune-compromised elderly patients. Unani medicines were correlated with the immune-stimulating activity. Antioxidant and immune-stimulating effect of *Aloe barbadensis* Mill, *Commiphora myrrh* (Nees) Engl. and *Crocus sativus* L., has already been established in animal models [16]. Research by Unani and Modern specialists on diverse plants has discovered a number of useful herbs that show the necessary pharmacotherapeutic actions that may be utilized for the treatment of HSV-1with the underlying properties of hydrochloric-inflammatory, anti-inflammatory, anti-pyretical, anti-hepatoprotective, cardiotonic and antiviral. Tiryaq-e-Wabai was supported in numerous areas in epidemics and pandemics by prominent doctors. Research on the preventive and supportive treatment of its

components has shown striking effects, further supported by exclusive Tiryaq-e-Wabai studies [17].

Materials and Methods:

Procurement and Preparation of Drug: Crude drugs were procured from a reputed herbal supplier and were identified and authenticated by a renowned botanist, C-RMR, Trans-Disciplinary University (TDU) Bengaluru, India.

Crude Drugs	Scientific name	Identification no
Sibr	*Aloe barbedensis* Mill	5560
Murmakki	*Commiphora myrrha* (Nees) Engl.	5561
Zaafran	*Crocus sativus* L.	5562

The drugs Sibr (*Aloe barbedensis* Mill): Murmakki (*Commiphora myrrha* (Nees) Engl.): Zafraan (*Crocus sativa* L.) were taken in the ratio 2:1:1 (bayazkabir) and were powdered finely. All the three drugs were taken in a mixer and were grinded together until fine powder. Sieve no 100 was used. The powder was stored in air tight glass jar, for further purposes.

Chemicals and Reagents:

Reagents: Sodium bicarbonate (MP Biomedicals, Lot No: 2048J), EDTA (MP Biomedicals, Lot No: 6941H), Trypan blue (Hy clone, Lot no: JRH27098), SRB Dye, DPBS (Dulbecco's phosphate buffer saline) (MP Biomedicals, Lot No: C1290), Trypsin (Invitrogen, Lot No: 1376596), MTT Salt.

Media: DMEM (Dulbecco's Modified Eagle's medium, high glucose), DMEM (Dulbecco's Modified Eagle's medium, low glucose), FBS (Foetal Bovine Serum) (Bio clot, Lot No: 07310).

Glass Wares: 96-well microtitre plate, Tissue culture flasks, Falcon tubes, Reagent bottles.

Kit for Cell proliferation: MTT (Roche applied sciences, Cat. No. 11 465 007 001).

Outline of the Method: Vero cells are produced from an African green monkey's kidney and are among the most popular continuous mammalian cell lines in microbiology and cell and molecular biology. Further, using two different non-toxic concentrations of the TW, viral inhibitory was tested against HSV- I. Vero cells are generated from normal kidney cells; they did not lose contact inhibition because cells are not altered. When cells meet, they cease growing and start to die; consequently, monitoring and culturing vero cells, as they form contiguous monolayers, is highly critical. The cell line was procured from NIV, Pune [18].

Preparation of TW for Stock Solution: The weighed TW was dissolved separately in DMSO as it has varying cellular and behavioural effects ranging from increased membrane permeability to toxicity, DMSO as a vehicle for microinjection could confound the effects of other drugs. The volume was made up of minimal essential medium (MEM) supplemented with 2% inactivated foetal bovine serum (FBS) to obtain a stock solution of 10 mg/ml concentration and sterilized by filtration. For carrying out cytotoxic studies, Serial two-fold dilutions were prepared.

Cell Line and Culture Medium: In MEM media supplemented with 10% inactivated Foetal Bovine Serum (FBS), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) penicillin (100 IU/ml), at 37°C in a humidified atmosphere of 5% CO_2 until confluent Vero cell line (Green monkey kidney) was cultured. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). In 25 cm2 culture flasks, stock cultures were grown, and then in 96 well microtitre plates, all experiments were carried out.

HSV-I was infected on to Vero cells followed by the freeze-thaw cycle of culture flasks to release the viral contents from the cells. Later the recovered HSV-1 was titrated and diluted to obtain the required challenge dose [19].

***In-vitro* Cytotoxicity Assay:** The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using MEM containing 10% FBS. 0.1 ml of the diluted cell suspension was added to each 96 well microtitreplate. When a partial monolayer was formed, After 24 h, the supernatant was removed quickly, then washed with medium monolayer once; subsequently, 100µl of different test concentrations of TW were added to the partial monolayer intomicrotitre plates. The plates were then incubated at 37°C for 72 h in a 5% CO2

atmosphere, microscopic examination was carried out, and observations were noted for every 24 h interval. After 72 h, 50 µl of MTT in PBS was added to each well after discarding the drug solutions. The plates were gently shaken and incubated for 3 h at 37°C in a 5% CO2 atmosphere to solubilize the formed formazan after removing supernatant by adding 100 µl of propanol and gently shaking the plates. The absorbance was measured at a wavelength of 540 nm by microplate reader. The percentage growth inhibition was calculated using standard formulae and concentration of TW needed to inhibit cell growth by 50% (CTC50) value was generated from the dose-response curves for the cell line [20].

Anti HSV-I Studies: Anti HSV-I activity of TW was evaluated by cytopathic effect (CPE) inhibition assay against 10TCID50 virus challenge doses of HSV-I. Prior to this, virus stock was standardized by titration. In the present experiment, the virus challenge dose (10TCID50) was prepared by suitable dilution technique (Reed–Muench method) and used as virus challenge doses against different TW [21].

CPE Inhibition Assay: Vero cells were grown in 96 well microtiter plates (2X104 cells/well) at 37°C in the presence of 5% CO_2. After 24h, when cells became confluent the culture medium was replaced with 0.1ml of virus suspension containing 10TCID50 challenge doses and incubated at 37°C for virus adsorption. After 60 min, cell supernatants were replaced with non-toxic concentrations of TW to appropriate wells in quadruplicate. Virus control and cell control were maintained and Acyclovir was used as a standard drug. The plates were incubated at 37 C with 5% CO_2 and were observed every 24h for CPE until 4-5th day post-infection. The presence or absence of CPE in each well was observed and scored depending on its degree of cytopathic effect. The activity of TW was determined by their inhibitory effect against viral cytopathicity and the reduction in the viral CPE by TW was expressed as percentage protection offered [22].

Virucidal Assay: Vero cells were cultivated as 1 × 105 cell/well in 96-well flat bottom culture plates in MEM culture medium at 37 °C in a humidified 5 % CO_2 atmosphere for 24 h. Different nontoxic concentrations of test drug, i.e., lower than CTC50 were tested for antiviral property by virucidal assay against virus challenge dose of 10 TCID50. The virus suspension (10 TCID50) with various concentrations of test Substance was incubated at 37°C for 1 hour (Test compound+ Virus suspension). In addition, the virus without test Substance was kept as virus control (Pathogen Control). After incubation 2.5% cell culture solution containing 10% inactivated foetal bovine serum was added into each tube to neutralize the test substance at room temperature. The neutralized solution was diluted 10 to 108 times with cell culture solution, and 100 µl of each mixture (Test compound+ Virus suspension) were added to the monolayer cultures grown in 96 well microtitre plates. The CPE was observed every 24 hours for 72 hours and compared with controls, which was expressed as the protection offered by the test samples to the cells was scored and the virus titre was estimated by endpoint titration method as TCID50/ml [23].

Statistical Analysis: STATA statistical analysis package was used for the dose-response curve drawing to IC_{50} and CC50 calculation [8].

RESULTS: The cell lines derived were free from contaminants. The percentage of cytotoxicity of the cell line was carried out using microtiter plate. The table shows that the percentage growth inhibition was calculated using standard formulae, and the concentration of TW needed to inhibit cell growth by 50% was greater than 2000 ug/ml. The Test Substance showed dose-dependent toxicity against Vero cells mentioned in **Table 1.**

Acyclovir is the first choice to treat HSV infections. The 75% of protection was achieved with the test concentration of 1000 ug/ml with TW; when compared with the Acyclovir concentration the effect achieved was 92%. The TW achieved 30% protection with half of its concentration of 500 ug/ml. Results were interpreted by comparing the titre obtained in the sets without antiviral agent with those obtained in the sets containing the antiviral agent. The virus suspension of 10TCID50 with various concentrations of the test substance was tested for virucidal activity. The TW powder at a concentration of 1000 ug/ml achieved a log reduction ratio of 2.17 where the acyclovir of 10

ug/ml achieved a log reduction of 4.33. For each experimental observation, the percentage of survival relative to the mean for the control (no virus) at each time point that is at 24, 48 and 72 hours was computed. An overall analysis of variance (ANOVA) with effect interactions were used to assess Survival rates by time, with radiation, without radiation and different viral MOIs. An indicator of a departure from the addition of effects would be bya significant two-way interaction with a virus, suggesting synergy. Cell survival was significantly reduced when the sample and virus were compared separately.

TABLE 1: CYTOTOXIC PROPERTIES OF TEST SUBSTANCE AGAINST VERO CELL LINE

Sl. no.	Name of Test Sample	Test Conc. (ug/ml)	% Cytotoxicity	CTC_{50} (ug/ml)
1	TW powder	2000	44.31±0.6	
		1000	24.48±0.7	
		500	20.85±1.6	>2000
		250	17.13±0.5	
		125	16.20±0.6	
		62.5	10.27±2.2	

TABLE 2: CYTOPATHIC INHIBITORY ACTIVITY OF TEST SUBSTANCE AGAINST HSV-I

Virus	Name of Test Substance	Viral load (TCID)	Test Conc. (µg/ml)	% Protection
			1000	75.00±4.45
HSV - I	TW powder	10	500	30.73±2.72
	Acyclovir	10	10	92.73±1.16

TABLE 3: VIRUCIDAL ACTIVITY OF TEST SUBSTANCE AGAINST HSV-I

Virus	Name of Test Substance	Test Conc. (µg/ml)	$TCID_{50}$	Log reduction
HSV - I	TW Powder	1000	4.0	2.17
		500	4.67	1.50
	Acyclovir (STD)	10	1.83	4.33
	Pathogen Control	---	6.17	-----

DISCUSSION: Unani medicine is one of the traditional medical systems being researched to ensure that patients receive preventative, supportive, and rehabilitative treatment. Unani's medical system thoroughly explains medications used in many infectious illnesses, including respiratory infections. The immune response is important to remove the virus and prevent the development to serious stages of illness. Strategies to stimulate immunological response are therefore absolutely significant. For that reason, it is necessary to outline the data on preventative measures, control alternatives like immune-stimulatory and prophylactic therapy for HSV-1through Unani medicine. Herpes simplex virus is one of the most common viral infection causing many diseases. The cell cytotoxic concentration (CC50) of this extract was several magnitudes higher than the effective concentrations inhibiting plaque formation by 50% (EC50), indicating the high safety margin of the extract. The virus yield reduction assay which simulates an in vivo environment of infection, demonstrated that the extract had a virucidal activity, further supporting the potency of the extract. The observations of the plaque inhibition assay on anti-HSV were also confirmed by virus yield reduction assay. An effective antiviral drug should have antiviral action, irreversibly block viral synthesis, and restore normal cell synthesis. In addition to this inhibition, the antiviral agent must have a broad spectrum of activity, favourable pharmacodynamic properties and not be immunosuppressive [21].

The findings outlined above indicate the Antiviral activity of the Unani test drug Tiryaqwabai and suggest its use in viral infections both in prophylactic and therapeutic. Currently, for SARS-CoV-2 infection no specific treatment has been identified. Natural substances such as herbs have previously demonstrated both great antiviral and anti-inflammatory activity. Thus, effective treatments against COVID-19 may seem promising and possible by natural substances. One of the potential candidates against the SARS-CoV-2 virus may be TW. We have evaluated the most effective herbal formulation in terms of the antiviral and

anti-inflammatory effects assessed in laboratory conditions.

CONCLUSION: The emphasis on health conservation and illness prevention was placed in Unani medicine. Although non-drug prophylaxis is the primary asset of Unani medicine, its use of preventive medicines in the pursuit of Taqaddumbil Hifz is not undermined. Innate heat stimulation and the increase of vital organ strength constitute a key basis for system illness prevention. One of the formulations under this category is Tiryaqewabai. It contains three components, Mur Makki *(Commiphora myrrha (*Nees) Engl.), Zafran (*Crocus sativus.* L) and Sibrzard (*Aloe barbadensis* Mill.) The substance has been shown to be effective for infectious diseases such as the plague, chicken pox, cholera and HSV-1. Tiryaqwabai has all three components which give a good basis for the prophylactic uses of in infectious state with diverse pharmacological actions such as immunomodulatory, atithusive, expectorative, and antiviral activity. Recent research has identified Tiryaqwabai's immune-stimulatory action and supports its application in diseases requiring immunostimulation. In addition, research is undergoing to treat Covid-19 with this crucial Unani prophylactic formula tiryaq e wabai. More research thrust in the Unani system of medicine would stimulate the commerce and practice of herbal items but helps to propagate Indian expertise throughout other areas of the world.

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